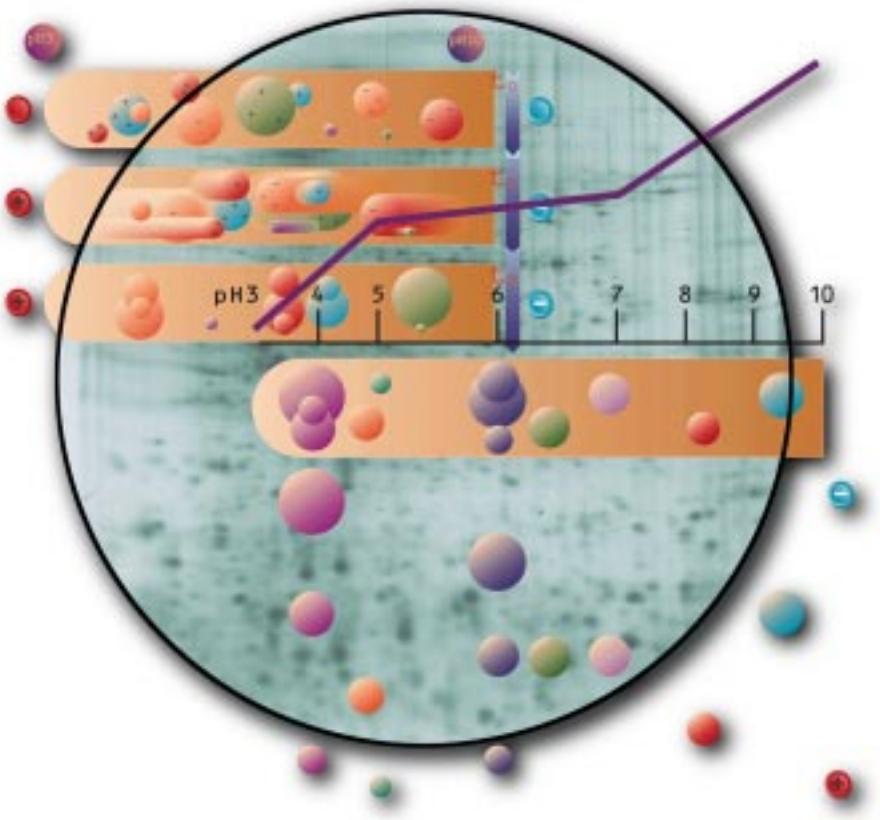
2-D Electrophoresis

USING IMMOBILIZED PH GRADIENTS



PRINCIPLES & METHODS

80–6429–60
Rev A / 9–98

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PRINCIPLES & METHODS

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Preface

"Proteomics" is the large-scale screening of the proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, spot identification, and database searches. The core technology of proteomics is 2-D electrophoresis. At present, there is no other technique which is capable of simultaneously resolving thousands of proteins in one separation procedure.

The replacement of classical first-dimension carrier ampholyte pH gradients with well-defined immobilized pH gradients has resulted in higher resolution, improved interlaboratory reproducibility, higher protein loading capacity, and an extended basic pH limit for 2-D electrophoresis. With the increased protein capacity, micropreparative 2-D electrophoresis has accelerated spot identification by mass spectrometry and Edman sequencing. With immobilized gradients stable as high as pH 12, basic proteins can be separated routinely where previously they were lost due to cathodic drift of carrier ampholyte gradients, or suffered from the limited reproducibility of NEPHGE.

The remarkable improvements in 2-D electrophoresis resulting from immobilized pH gradient gels, together with convenient new instruments for IPG-IEF, will make critical contributions to advances in proteome analysis.

It is my pleasure to introduce this manual on 2-D electrophoresis. It clearly describes the actual and technical basis of the current state-of-the-art 2-D separations using immobilized pH gradients for the first dimension, it provides detailed protocols for new and experienced users, and it includes an extensive bibliography. Finally, there is the pictorial troubleshooting guide—a bit like photos from the album of Murphy's law that you *wouldn't dare* include in an official publication—but here they are for all to learn from.

Angelika Görg

Technical University of Munich, August 1998

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Introduction

1.0 Introduction to the manual

This manual is divided into four parts. Part I provides guidelines for sample preparation. Part II details procedures for performing the first dimension of 2-D electrophoresis. Part III contains general directions for subsequent second-dimension electrophoresis of IPG strips. Part IV discusses visualization and analysis of the 2-D electrophoresis results. The 2-D protocols described herein use products of Amersham Pharmacia Biotech. Equipment choices are discussed in section 1.2.

1.1 Introduction to two-dimensional (2-D) electrophoresis

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (MW). Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtained.

Two-dimensional electrophoresis was first introduced by P.H. O'Farrell [1] and J. Klose [2] in 1975. In the original technique, the first-dimension separation was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. (Under the influence of an electric current, carrier ampholytes form a pH gradient, a critical component of IEF. See section 3.1, 'Background to IEF,' for more detail.) Sample was applied to one end of each tube gel and separated at high voltages. After IEF the gel rods were removed from their tubes, equilibrated in SDS sample buffer, and placed on vertical SDS-polyacrylamide gels for the second-dimension separation.

The power of 2-D electrophoresis as a biochemical separation technique has been recognized virtually since its introduction. Its application, however, has become significant only in the past few years as a result of a number of developments.

- The 2-D technique has been improved to generate 2-D maps that are superior in terms of resolution and reproducibility. This new 2-D technique, developed by A. Görg and colleagues [3,4], utilizes an improved first-dimension separation method that replaces the carrier ampholyte-generated pH gradients with immobilized pH gradients (IPG) and replaces the tube gels with gel strips supported by a plastic film backing. A more detailed discussion of the merits of this technique is presented in section 3.1, 'Background to IEF.'
- Methods for the rapid analysis of proteins have been improved to the point that single spots eluted or transferred from single 2-D gels can be rapidly identified. Mass spectroscopic techniques have been developed that allow analysis of very small quantities of peptides and proteins. Chemical microsequencing and amino acid analysis can be performed on increasingly smaller samples. Immunochemical identification is now possible with a wide assortment of available antibodies.
- More-powerful, less expensive computers and software are now available, allowing routine computerized evaluations of the highly complex 2-D patterns.
- Data about entire genomes (or substantial fractions thereof) for a number of organisms are now available, allowing rapid identification of the gene encoding a protein separated by 2-D electrophoresis.
- The World Wide Web provides simple, direct access to spot pattern databases for the comparison of electrophoresis results and to genome sequence databases for assignment of sequence information.

A large and growing application of 2-D electrophoresis is "proteome analysis." Proteome analysis is "the analysis of the PROTEin complement expressed by a genOME" [5,6]. The analysis involves the systematic separation, identification, and quantification of many proteins simultaneously from a single sample. 2-D electrophoresis is used in this application due to its unparalleled ability to separate thousands of proteins. 2-D electrophoresis is also unique in its ability to detect post- and cotranslational modifications, which cannot be predicted from the genome sequence.

Other applications of 2-D electrophoresis include analysis of cell differentiation, detection of disease markers, monitoring therapies, drug discovery, cancer research, purity checks, and microscale protein purification.

This manual describes methods for 2-D electrophoresis using precast IPG strips (Immobiline® DryStrip gels) available from Amersham Pharmacia Biotech. The 2-D process begins with sample preparation. Proper sample

TABLE 1. EQUIPMENT CHOICES FOR 2-D ELECTROPHORESIS

Choices for first-dimension IEF

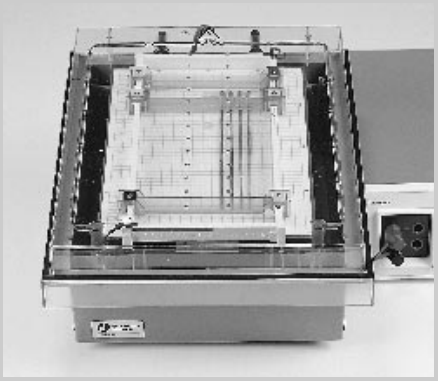


Figure 1. Multiphor II Electrophoresis unit with Immobiline DryStrip Kit

Multiphor® II Electrophoresis unit with Immobiline® DryStrip Kit

Rehydration in Reswelling Tray

↓

IEF in Multiphor II unit with Immobiline DryStrip Kit

Choice Factors:

- Multiphor II can be used for both first- and second-dimension separations.
- Multiphor II is a versatile system. Its use is not limited to IEF with IPG strips. Several different electrophoresis techniques can be performed with the instrument.

Figure 2. IPGphor Isoelectric Focusing System

IPGphor™ Isoelectric Focusing System

Rehydration and IEF, both in IPGphor strip holder

Choice Factors:

- Rehydration and IEF can be performed overnight, unattended.
- Fewer IPG strip manipulations are required, reducing the chance of error.
- Faster separations and sharper focusing are possible because of higher voltage.
- Power supply and temperature control are built into the instrument.

TABLE 1. EQUIPMENT CHOICES FOR 2-D ELECTROPHORESIS (continued)

Choices for second-dimension SDS-PAGE

Figure 3. Multiphor II flatbed system

Multiphor II (flatbed system)
24.5 × 11 cm or 24.5 × 18 cm gels

Choice Factors:

- Precast gels offered:
  ExcelGel® 8–18% (24.5 × 11 cm), ExcelGel XL 12–14% (24.5 × 18 cm).
- Relatively rapid: 4 hours or less for electrophoresis.
- High resolution.
- All available IPG strip lengths can be used.

Figure 4. Hoefer miniVE

Hoefer® miniVE or SE 260 (mini vertical)
8 × 9 cm gels

Choice Factors:

- Rapid: 1–2 hours for electrophoresis.
- Best for 7 cm IPG strips.

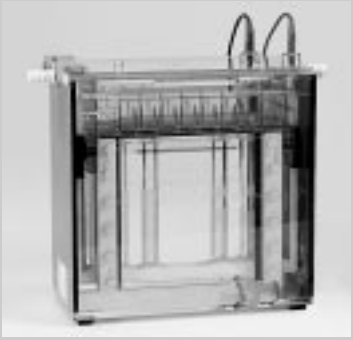


Figure 5. Hoefer SE 600

Hoefer® SE 600 (standard vertical)
14 (or 16) × 15 cm gels

Choice Factors:

- 4–5 hours for electrophoresis.
- Intermediate separation (15 cm gel length).
- Intermediate throughput (up to four gels simultaneously).
- Best for 13 cm IPG strips.

Figure 6. Hoefer DALT

Hoefer® DALT (large-format vertical)
24 × 19 cm gels

Choice Factors:

- 7 hours to overnight electrophoresis.
- Highest resolution (19 cm gel length).
- Highest possible protein capacity.
- High throughput (up to 10 gels simultaneously).
- Best for 18 cm IPG strips.

preparation is absolutely essential for a good 2-D result. The next step in the 2-D process is IPG strip rehydration. IPG strips are provided dry and must be rehydrated with the appropriate additives prior to IEF. First-dimension IEF is performed on a flatbed system at very high voltages with active temperature control. Next, strip equilibration in SDS-containing buffer prepares the sample for the second-dimension separation. Following equilibration, the strip is placed on the second-dimension gel for SDS-PAGE. The final steps are visualization and analysis of the resultant two-dimensional array of spots.

In summary, the experimental sequence for 2-D electrophoresis is:

1. Sample preparation
2. IPG strip rehydration
3. IEF
4. IPG strip equilibration
5. SDS-PAGE
6. Visualization
7. Analysis

1.2 Equipment choices

Different options exist in terms of methods and equipment for IEF and SDS-PAGE. Table 1 lists the instruments available from Amersham Pharmacia Biotech. For detailed information on the operation of any of the instruments described, please see the respective User Manual.

Selecting an IEF system

Amersham Pharmacia Biotech offers two different systems for the first-dimension separation: the Multiphor® II system with associated accessories, and the IPGphor™ Isoelectric Focusing System.

Multiphor II is a versatile system that can be used for several different electrophoresis techniques. For 2-D electrophoresis it can be used for both first-dimension IEF and second-dimension SDS-PAGE. Strip rehydration is performed in the Immobiline DryStrip Reswelling Tray. After rehydration the IPG strips are transferred to the electrophoresis unit for first-dimension IEF. The electrophoresis system is comprised of the Multiphor II flatbed unit with the Immobiline DryStrip Kit (Figure 1). This system accommodates up to 12 rehydrated IPG strips of the same length for any one IEF protocol. Power is supplied by the EPS 3501 XL power supply, and temperature control is provided by the MultiTemp® III Thermostatic Circulator.

The IPGphor Isoelectric Focusing System (Figure 2) further simplifies the first-dimension separation with a system dedicated to IEF separation on IPG strips. The system is comprised of IPG strip holders that serve as both rehydration and IEF chambers, and the IPGphor unit, which includes an 8,000 V power supply and built-in temperature control. Programmable parameters include rehydration temperature and duration, IEF temperature and maximum current, and the duration and voltage pattern of multiple steps for one separation. Up to 12 strip holders of the same length can be placed on the IPGphor platform for any one protocol. Because rehydration and IEF are performed consecutively without user intervention, they can be performed unattended overnight. Fewer IPG strip manipulations result in less error, strip mix-up, contamination, air contact, and urea crystallization. Separations are faster because of the substantially higher voltage that can be applied.

Table 2 shows the key operating differences between the Multiphor II system and the IPGphor Isoelectric Focusing System for first-dimension IEF.

TABLE 2. IEF SYSTEM SELECTION

	Maximum voltage	Additional equipment required	Time required for IEF[1]
Multiphor II	3500 V[2]	Immobiline DryStrip Reswelling Tray, Immobiline DryStrip Kit, EPS 3501 XL power supply, MultiTemp III Thermostatic Circulator	3–48 hours
IPGphor	8000 V	IPG strip holders of desired length	1.5–24 hours

[1] Optimal focusing time varies widely depending on the IPG strip length and pH range and the nature of the sample. Similar separations can generally be performed at least twofold faster with the IPGphor system than with the Multiphor II system.

[2] Higher voltages are not recommended for safety reasons.

Selecting a second-dimension system

The second-dimension separation may be performed in a vertical or flatbed system. Table 3 matches the appropriate second-dimension system and gel size with IPG strip length. Further considerations are discussed below. (For a more complete discussion of the relative merits of flatbed vs. vertical second dimensions, consult [7].)

TABLE 3. SECOND-DIMENSION ELECTROPHORESIS SYSTEM SELECTION

	Approx. gel size (wxl, cm)	Number of gels	Gel thickness (mm)	IPG strip length (cm)	Total oper. time (h:min)
Flatbed					
Multiphor II, ExcelGel	24.5 × 11, 24.5 × 18	1[1]	0.5	all	1:45 3:20
Vertical					
Hoefer miniVE or SE 260	8 × 9	2	1, 1.5	7	1:30
Hoefer SE 600	14 × 15, 16 × 15[2]	2 or 4[3]	1, 1.5	11, 13	5:00
Hoefer DALT	24 × 19	10	1, 1.5	18	7:00–15:00

[1] Multiple shorter IPG strips fit on one ExcelGel: two 11 cm strips or three 7 cm strips.
[2] If 1-cm-wide spacers are used.
[3] Accessory divider plates increase the capacity to four gels.

Multiphor II flatbed system

This system provides excellent resolution and relatively rapid separations in a large-format gel. Precast ExcelGel products offer the convenience of ready-to-use gels and buffer strips.

The Multiphor II system (Figure 3) offers convenience and versatility as it can be used for both first-dimension IEF as well as second-dimension SDS-PAGE.

The protein loading capacity of an IPG strip can exceed the capacity of the thin, horizontal second-dimension gel, so thicker vertical second-dimension gels are preferred for micro-preparative separations.

The Multiphor II system is not recommended for the second dimension if pH 6–11 IPG strips have been used for the first-dimension separation.

Vertical systems

Vertical systems offer relative ease of use and the possibility of performing multiple separations simultaneously. Vertical 2-D gels can be either 1 or 1.5 mm thick.

For rapid results, the mini-gel units—the Hoefer miniVE (Figure 4) or the SE 260—are recommended. The second-dimension separation is typically complete in 1 to 2 hours. The use of mini-gels for the second dimension of 2-D is ideal when quick profiling is required or when there are relatively few different proteins in the sample.

For increased throughput and resolution, the standard-sized SE 600 vertical gel system (Figure 5) is recommended. The SE 600 accommodates up to four 16-cm-long gels, and the built-in heat exchanger offers cooling capability for increased reproducibility when used with a thermostatic circulator such as MultiTemp III. The standard spacer width is 2 cm, giving a 14-cm-wide gel. If additional space for molecular weight markers is desired at both ends of a 13 cm IPG strip, 1-cm-wide spacers are available for the preparation of 16-cm-wide gels.

For maximal resolution, reproducibility, and capacity, the large-gel format of the Hoefer DALT system (Figure 6) is recommended. The Hoefer DALT system can accommodate the entire gradient of an 18 cm IPG strip (plus molecular weight markers) and up to 10 gels can be run simultaneously. A built-in heat exchanger and buffer circulation pump provide precise temperature control and a uniform thermal environment. Twenty or more 1- or 1.5-mm-thick gels can be cast simultaneously in the Hoefer DALT Multiple Gel Caster.

1.3 Laboratory technique

- Always wear gloves when handling IPG strips, SDS polyacrylamide gels, ExcelGel Buffer Strips, and any equipment that these items will contact. The use of gloves will reduce protein contamination that can produce spurious spots or bands in 2-D patterns.
- Clean all assemblies that will contact the gels or sample with a detergent designed for glassware and rinse well with distilled water.
- Always use the highest-quality reagents and the purest water available.

Part I

Sample preparation

2.0 Sample preparation — general strategy

Appropriate sample preparation is absolutely essential for good 2-D results. Due to the great diversity of protein sample types and origins, only general guidelines for sample preparation are provided in this guide. The optimal procedure must be determined empirically for each sample type. Ideally, the process will result in the complete solubilization, disaggregation, denaturation, and reduction of the proteins in the sample.

When developing a sample preparation strategy, it is important to have a clear idea of what is desired in the final 2-D result. Is the goal to view as many proteins as possible, or is only a subset of the proteins in the sample of potential interest? Which is more important, complete sample representation, or a clear, reproducible pattern? Additional sample preparation steps can improve the quality of the final result, but each additional step can result in the selective loss of protein species. The trade-off between improved sample quality and complete protein representation must therefore be carefully considered.

In order to characterize specific proteins in a complex protein mixture, the proteins of interest must be completely soluble under electrophoresis conditions. Different treatments and conditions are required to solubilize different types of protein samples: some proteins are naturally found in complexes with membranes, nucleic acids, or other proteins; some proteins form various non-specific aggregates; and some proteins precipitate when removed from their normal environment. The effectiveness of solubilization depends on the choice of cell disruption method, protein concentration and dissolution method, choice of detergents, and composition of the sample solution. If any of these steps is not optimized for a particular sample, separations may be incomplete or distorted and information may be lost.

To fully analyze all intracellular proteins, the cells must be effectively disrupted. Choice of disruption method depends on whether the sample is from cells, solid tissue, or other biological material and whether the analysis is targeting all proteins or just a particular subcellular fraction. Both gentle and vigorous lysis methods are discussed in section 2.1.

Proteases may be liberated upon cell disruption. Proteolysis greatly complicates analysis of the 2-D result, thus the protein sample should be protected from proteolysis during cell disruption and subsequent preparation. Protease inhibition is discussed in section 2.2.

If only a subset of the proteins in a tissue or cell type is of interest, prefractionation can be employed during sample preparation. If proteins from one particular subcellular compartment (e.g., nuclei, mitochondria, plasma membrane) are desired, the organelle of interest can be purified by differential centrifugation or other means prior to solubilization of proteins for 2-D electrophoresis. The sample can also be prefractionated by solubility under different extraction conditions prior to 2-D electrophoresis. References [8,9] describe examples of this approach. See reference [10] for an overview of the subject of protein fractionation.

Precipitation of the proteins in the sample and removal of interfering substances are optional steps. The decision to employ these steps depends on the nature of the sample and the experimental goal. Precipitation procedures, which are used both to concentrate the sample and to separate the proteins from potentially interfering substances, are described in section 2.3. Removal techniques, which eliminate specific contaminants from the sample, are described in section 2.4, as are the effects contaminants (salts, small ionic molecules, ionic detergents, nucleic acids, polysaccharides, lipids, and phenolic compounds) might have on the 2-D result if they are not removed.

In general, it is advisable to keep sample preparation as simple as possible. A sample with low protein concentrations and a high salt concentration, for example, could be diluted normally and analyzed, or desalted, then concentrated by lyophilization, or precipitated with TCA and ice-cold acetone and re-solubilized with rehydration solution. The first option of simply diluting the sample with rehydration solution may be sufficient. If problems with protein concentration or interfering substances are otherwise insurmountable, precipitation or removal steps may be necessary.

The composition of the sample solution is particularly critical for 2-D, because solubilization treatments for the first-dimension separation must not affect the protein pI, nor leave the sample in a highly conductive solution. In general, concentrated urea as well as one or more detergents are used. Sample solution composition is discussed in section 2.5.

General sample preparation guidelines:

- Keep the sample preparation strategy as simple as possible to avoid protein losses. Additional sample preparation steps may improve the quality of the final 2-D result, but at the possible expense of selective protein loss.
- The cells or tissue should be disrupted in such a way as to minimize proteolysis and other modes of protein degradation. Cell disruption should be done at as low a temperature as possible and with a minimum of heat generation. Cell disruption should ideally be carried out directly into a strongly denaturing solution containing protease inhibitors.
- Sample preparation solutions should be freshly prepared or stored as frozen aliquots. Use high-purity or de-ionized urea.
- Preserve sample quality by preparing the sample just prior to IEF or storing samples in aliquots at –80 °C. Do not expose samples to repeated thawing.
- Remove all particulate material by ultracentrifugation. Solid particles and lipids must be removed because they will block the gel pores.
- To avoid modification of proteins, never heat a sample after adding urea. When the sample contains urea, it must not be heated over 37 °C. Elevated temperatures cause urea to hydrolyze to isocyanate, which modifies proteins by carbamylation.
- For more-specific guidance on preparing samples for application to IPG strips see [11–13].

2.1 Methods of cell disruption

Listed in Tables 4 and 5 are a few standard disruption methods, both mechanical and chemical. Cell disruption should be performed at cold temperatures. Keep the sample on ice as much as possible and use chilled solutions. Proteases may be liberated upon cell disruption, thus the protein sample should be protected from proteolysis if one of these methods is to be used. (See section 2.2.) It is generally preferable to disrupt the sample material directly into a strongly denaturing lysis solution in order to rapidly inactivate proteases and other enzymatic activities that may modify proteins. Cell disruption is often carried out in an appropriate solubilization solution for the proteins of interest. References [14,15] contain general information on tissue disruption and cell lysis.

2.1.1 Gentle lysis methods

Gentle lysis methods, listed in Table 4, are generally employed when the sample of interest consists of easily

TABLE 4. GENTLE LYSIS METHODS

Cell disruption method	Application	General procedure
Osmotic lysis [16] This very gentle method is well suited for applications in which the lysate is to be subsequently fractionated into subcellular components.	Blood cells, tissue culture cells	Suspend cells in a hypoosmotic solution.
Freeze-thaw lysis [8,14,17] Many types of cells can be lysed by subjecting them to one or more cycles of quick freezing and subsequent thawing.	Bacterial cells, tissue culture cells	Rapidly freeze cell suspension using liquid nitrogen, then thaw. Repeat if necessary.
Detergent lysis Detergents solubilize cellular membranes, lysing cells and liberating their contents.	Tissue culture cells	Suspend cells in lysis solution containing detergent. Cells can often be lysed directly into sample solution or rehydration solution because these solutions always contain detergent. See Appendix, solution A, for an example of a widely used lysis solution. Further examples of this technique are given in [18,19]. If an anionic detergent such as SDS is used for lysis, one of the following preparation steps is required to ensure that the SDS will not interfere with IEF: • Dilute the lysed sample into a solution containing an excess of non-ionic or zwitterionic detergent. • Or, separate the SDS from the sample protein by acetone precipitation. (See Tables 7 and 8 and section 2.5 for details.)
Enzymatic lysis [20,21] Cells with cell walls can be lysed gently following enzymatic removal of the cell wall. This must be done with an enzyme specific for the type of cell to be lysed (e.g., lysozyme for bacterial cells, cellulase and pectinase for plant cells, lyticase for yeast cells).	Plant tissue, bacterial cells, fungal cells	Treat cells with enzyme in isoosmotic solution.

lysed cells (such as tissue culture cells, blood cells, and some microorganisms). Gentle lysis methods can also be employed when only one particular subcellular fraction is to be analyzed. For example, conditions can be chosen in which only cytoplasmic proteins are released, or intact mitochondria or other organelles are recovered by differential centrifugation. Sometimes these techniques are combined (e.g., osmotic lysis following enzymatic treatment, freeze-thaw in the presence of detergent).

2.1.2 More-vigorous lysis methods

More-vigorous lysis methods, listed in Table 5, are employed when cells are less easily disrupted, i.e., cells in solid tissues or cells with tough cell walls. More-vigorous lysis methods will result in complete disruption of the cells, but care must be taken to avoid heating or foaming during these procedures.

TABLE 5. MORE-VIGOROUS LYSIS METHODS

Cell disruption method	Application	General procedure
Sonication [4,22,23] Ultrasonic waves generated by a sonicator lyse cells through shear forces. Complete shearing is obtained when maximal agitation is achieved, but care must be taken to minimize heating and foaming.	Cell suspensions	Sonicate cell suspension in short bursts to avoid heating. Cool on ice between bursts.
French pressure cell [20,21,24] Cells are lysed by shear forces resulting from forcing cell suspension through a small orifice under high pressure.	Microorganisms with cell walls (bacteria, algae, yeasts)	Place cell suspension in chilled French pressure cell. Apply pressure and collect extruded lysate.
Grinding [4,7,25,26] Some cell types can be opened by hand grinding with a mortar and pestle.	Solid tissues, microorganisms	Tissue or cells are normally frozen with liquid nitrogen and ground to a fine powder. Alumina or sand may aid grinding.
Mechanical homogenization [8,16,27–29] Many different devices can be used to mechanically homogenize tissues. Handheld devices such as Dounce or Potter-Elvehjem homogenizers can be used to disrupt cell suspensions or relatively soft tissues. Blenders or other motorized devices can be used for larger samples. Homogenization is rapid and poses little danger to proteins except by the proteases that may be liberated upon disruption.	Solid tissues	Chop tissue into small pieces if necessary. Add chilled homogenization buffer (3–5 volumes to volume of tissue). Homogenize briefly. Clarify lysate by filtration and/or centrifugation.
Glass bead homogenization [20,21,30] The abrasive actions of the vortexed beads break cell walls, liberating the cellular contents.	Cell suspensions, microorganisms	Suspend cells in an equal volume of chilled lysis solution and place into a sturdy tube. Add 1–3 grams of chilled glass beads per gram of wet cells. Vortex 1 minute and incubate cells on ice 1 minute. Repeat vortexing and chilling two to four times.

2.2 Protection against proteolysis

When cells are lysed, proteases are often liberated or activated. Degradation of proteins through protease action greatly complicates the analysis of 2-D electrophoresis results, so measures should be taken to avoid this problem. If possible, inhibit proteases by disrupting the sample directly into strong denaturants such as 8 M urea, 10% TCA, or 2% SDS [31–35]. Proteases are less active at lower temperatures, so sample preparation at as low a temperature as possible is recommended. In addition, most tissue proteases are inactive above pH 9, so proteolysis can often be inhibited by preparing the sample in the presence of tris base, sodium carbonate, or basic carrier ampholyte mixtures.

These approaches alone are often sufficient protection against proteolysis. Some proteases, however, may retain some activity even under these conditions. In these cases, protease inhibitors may be used. Individual protease inhibitors are active only against specific classes of proteases, so it is usually advisable to use a combination of protease inhibitors. Broad-range protease inhibitor "cocktails" are available from a number of commercial sources. Table 6 lists common protease inhibitors and the proteases they inhibit. For more, comprehensive-discussions of protease inhibition, see [12,28,36–40].

TABLE 6. PROTEASE INHIBITORS

Protease inhibitor	Effective against	Limitations
PMSF (Phenylmethylsulphonyl fluoride) Most commonly used inhibitor. *Use at concentrations up to 1 mM.*	PMSF is an irreversible inhibitor that inactivates: • serine proteases • some cysteine proteases	PMSF rapidly becomes inactive in aqueous solutions: Prepare just prior to use. PMSF may be less effective in the presence of thiol reagents such as DTT or 2-mercaptoethanol. This limitation can be overcome by disrupting the sample into PMSF-containing solution lacking thiol reagents. Thiol reagents can be added at a later stage. PMSF is very toxic.
AEBSF (Aminoethyl benzylsufonyl fluoride or Pefabloc® SC) *Use at concentrations up to 4 mM.*	AEBSF is similar to PMSF in its inhibitory activity, but is more soluble and less toxic.	AEBSF-induced modifications can potentially alter the pI of a protein.
1 mM EDTA or 1 mM EGTA *Generally used at 1 mM.*	These compounds inhibit metalloproteases by chelating free metal ions required for activity.	
Peptide protease inhibitors (e.g., leupeptin, pepstatin, aprotinin, bestatin) These inhibitors are: • reversible inhibitors • active in the presence of DTT • active at low concentrations under a variety of conditions *Use at 2–20 µg/ml.*	Leupeptin inhibits many serine and cysteine proteases. Pepstatin inhibits aspartyl proteases (e.g., acidic proteases such as pepsin). Aprotinin inhibits many serine proteases. Bestatin inhibits aminopeptidases.	Peptide protease inhibitors are expensive. Peptide protease inhibitors are small peptides and thus may appear on the 2-D map, depending on the size range separated by the second-dimension gel. Pepstatin does not inhibit any proteases that are active at pH 9.
TLCK, TPCK (Tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone) *Use at 0.1–0.5 mM.*	These similar compounds irreversibly inhibit many serine and cysteine proteases.	
Benzamidine *Use at 1–3 mM.*	Benzamidine inhibits serine proteases.	

2.3 Precipitation procedures

Protein precipitation is an optional step in sample preparation for 2-D electrophoresis. Precipitation, followed by resuspension in sample solution, is generally employed to selectively separate proteins in the sample from contaminating species such as salts, detergents, nucleic acids, lipids, etc., that would otherwise interfere with the 2-D result. Precipitation followed by resuspension can also be employed to prepare a concentrated protein sample from a dilute source (e.g., plant tissues, urine).

No precipitation technique is completely efficient, and some proteins may not readily resuspend following precipitation. Thus, employing a precipitation step during sample preparation may alter the protein profile of a sample. Precipitation and resuspension should be avoided if the aim of a 2-D experiment is complete and accurate representation of all the proteins in a sample. Table 7 lists some of the precipitation techniques used. If sample preparation requires precipitation, typically only one precipitation technique is employed.

For an overview of precipitation techniques, see [14,15,41].

TABLE 7. PRECIPITATION PROCEDURES

Precipitation method	General procedure	Limitations
Ammonium sulphate precipitation ("Salting out") In the presence of high salt concentrations, proteins tend to aggregate and precipitate out of solution. Many potential contaminants (e.g., nucleic acids) will remain in solution.	Prepare protein so final concentration of the protein solution is >1 mg/ml in a buffer solution that is >50 mM and contains EDTA. Slowly add ammonium sulphate to the desired percent saturation [41] and stir for 10–30 minutes. Pellet proteins by centrifugation.	Many proteins remain soluble at high salt concentrations, so this method is not recommended when total protein representation is desired. This method can, however, be used for prefractionation or enrichment. Residual ammonium sulphate will interfere with IEF and must be removed [42]. See section 2.4 on removal of salts.
TCA precipitation TCA (trichloroacetic acid) is a very effective protein precipitant.	TCA is added to the extract to a final concentration of 10–20% and the proteins are allowed to precipitate on ice for 30 minutes [43]. Alternatively, tissue may be homogenized directly into 10–20% TCA [32,44]. This approach limits proteolysis and other protein modifications. Centrifuge and wash pellet with acetone or ethanol to remove residual TCA.	Proteins may be difficult to resolubilize and may not resolubilize completely. Residual TCA must be removed by extensive washing with acetone or ethanol. Extended exposure to this low-pH solution may cause some protein degradation or modification.
Acetone precipitation This organic solvent is commonly used to precipitate proteins. Many organic-soluble contaminants (e.g., detergents, lipids) will remain in solution.	Add at least 3 volumes of ice-cold acetone to the extract. Allow proteins to precipitate at –20 ºC for at least 2 hours. Pellet proteins by centrifugation [43,45–47]. Residual acetone is removed by air drying or lyophilization.	
Precipitation with TCA in acetone The combination of TCA and acetone is commonly used to precipitate proteins during sample preparation for 2-D electrophoresis and is more effective than either TCA or acetone alone.	Suspend lysed or disrupted sample in 10% TCA in acetone with either 0.07% 2-mercaptoethanol or 20 mM DTT. Precipitate proteins for at least 45 minutes at –20 ºC. Pellet proteins by centrifugation and wash pellet with cold acetone containing either 0.07% 2-mercaptoethanol or 20 mM DTT. Remove residual acetone by air drying or lyophilization [4,25,31,40,48,49].	Proteins may be difficult to resolubilize and may not resolubilize completely. Extended exposure to this low-pH solution may cause some protein degradation or modification.
Precipitation with ammonium acetate in methanol following phenol extraction This technique has proven useful with plant samples containing high levels of interfering substances.	Proteins in the sample are extracted into water- or buffer-saturated phenol. Proteins are precipitated from the phenol phase with 0.1 M ammonium acetate in methanol. The pellet is washed several times with ammonium acetate in methanol and then with acetone. Residual acetone is evaporated [40,39,44,50].	The method is complicated and time consuming.

2.4 Removal of contaminants that affect 2-D results

Non-protein impurities in the sample can interfere with separation and subsequent visualization of the 2-D result, so sample preparation can include steps to rid the sample of these substances. Table 8 lists contaminants that affect 2-D results and techniques for their removal. Reference [12] provides further discussion on the removal of interfering substances.

TABLE 8. CONTAMINANTS THAT AFFECT 2-D RESULTS

Contaminant	Reason for removal	Removal techniques
Salts, residual buffers, and other charged small molecules that carry over from sample preparation	Salts disturb the electrophoresis process and must be removed or maintained at as low a concentration as possible. Salts in the IPG strip result in high strip conductivity. Focusing of the proteins will not occur until the ions have moved to the ends of the strips, prolonging the time required for IEF. Water movement can also result, causing one end of the strip to dry out and the other to swell. Salt in the IPG strip can result in large regions at either end of the IPG strip where proteins do not focus (seen as horizontal streaking in the final result). If the sample is rehydrated into the IPG strip, the salt concentration in the rehydration solution should be lower than 10 mM. If the sample is applied in sample cups, salt concentrations of up to 50 mM in the sample may be tolerated; however, proteins may precipitate at the sample application point as they abruptly move into a lower-salt environment.	Desalting can be performed by: • dialysis • spin dialysis • gel filtration • precipitation/resuspension Dialysis is a very effective method for salt removal, resulting in minimal sample loss; however, the process is time consuming and requires large volumes of solution. Spin dialysis is quicker, but protein adsorption onto the dialysis membrane may be a problem. Spin dialysis should be applied to samples prior to addition of urea and detergent. Gel filtration can be acceptable but often results in protein losses. Precipitation/resuspension is an effective means of removing salts and other contaminants, but can also result in losses (see section 2.3).
Endogenous small ionic molecules (nucleotides, metabolites, phospholipids, etc.)	Endogenous small ionic molecules are present in any cell lysate. These substances are often negatively charged and can result in poor focusing toward the anode.	TCA/acetone precipitation is particularly effective at removing this sort of contaminant. Other desalting techniques may be applied (see above).
Ionic detergent	Ionic detergent (usually SDS) is often used during protein extraction and solubilization, but can strongly interfere with IEF. SDS forms complexes with proteins, and the resulting negatively charged complex will not properly focus unless the SDS is removed or sequestered.	Dilute the SDS-containing sample into a rehydration solution containing a zwitterionic or non-ionic detergent (CHAPS, Triton X-100, or NP-40) so the final concentration of SDS is 0.25% or lower and the ratio of the other detergent to SDS is at least 8:1 [24]. Acetone precipitation of the protein will partially remove SDS. Precipitation at room temperature will maximize removal of SDS, but protein precipitation is more complete at –20 ºC [43].
Nucleic acids (DNA, RNA)	Nucleic acids increase sample viscosity and cause background smears. High-molecular-weight nucleic acids can clog gel pores. Nucleic acids can bind to proteins through electrostatic interactions, preventing focusing. If the separated sample proteins are visualized by silver staining, nucleic acids present in the gel will also stain, resulting in a background smear on the 2-D gel.	Treat samples rich in nucleic acids with a protease-free DNase/RNase mixture to reduce the nucleic acids to mono- and oligonucleotides. This is often done by adding 0.1 × volume of a solution containing 1 mg/ml DNase I, 0.25 mg/ml RNase A, and 50 mM $MgCl_2$ followed by incubation on ice [30,47]. *Note:* The DNase and RNase proteins may appear on the 2-D map. Ultracentrifugation can be used to remove large nucleic acids; however, this technique may also remove high-molecular-weight proteins from the sample. When using low-ionic-strength extraction conditions, negatively charged nucleic acids may form complexes with positively charged proteins. High-ionic-strength extraction and/or high-pH extraction may minimize these interactions. (Note that salts added during extraction must be subsequently removed; see above.)

continues on following page

TABLE 8. CONTAMINANTS THAT AFFECT 2-D RESULTS (continued)

Contaminant	Reason for removal	Removal techniques
Polysaccharides	Polysaccharides can clog gel pores, causing either precipitation or extended focusing times and resulting in horizontal streaking. Some polysaccharides contain negative charges and can complex with proteins by electrostatic interactions.	Precipitate the sample in TCA, ammonium sulphate, or phenol/ammonium acetate, then centrifuge. Ultracentrifugation will remove high-molecular-weight polysaccharides. Employing the same methods used for preventing protein-nucleic acid interactions may also be helpful (solubilize sample in SDS or at high pH).
Lipids	Many proteins, particularly membrane proteins, are complexed with lipids. This reduces their solubility and can affect both the pI and the molecular weight. Lipids form complexes with detergents, reducing the effectiveness of the detergent as a protein solubilizing agent. When extracts of lipid-rich tissues are centrifuged, there is often a lipid layer that can be difficult to remove.	Strongly denaturing conditions and detergents minimize protein-lipid interactions. Excess detergent may be necessary. Precipitation with acetone removes some lipid.
Phenolic compounds	Phenolic compounds are present in many plant tissues and can modify proteins through an enzyme-catalyzed oxidative reaction [40,46].	Prevent phenolic oxidation by employing reductants during tissue extraction (e.g., DTT, 2-mercaptoethanol, sulphite, ascorbate). Rapidly separate proteins from phenolic compounds by precipitation techniques. Inactivate polyphenol oxidase with inhibitors such as diethyldithiocarbamic acid or thiourea. Remove phenolic compounds by adsorption to polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP).
Insoluble material	Insoluble material in the sample can clog gel pores and result in poor focusing. Insoluble material is particularly problematic when the sample is applied using sample cups: It can prevent protein entry into the IPG strip.	Samples should always be clarified by centrifugation prior to application to first-dimension IEF.

2.5 Composition of sample solution

In order to achieve a well-focused first-dimension separation, sample proteins must be completely disaggregated and fully solubilized. Regardless of whether the sample is a relatively crude lysate or additional sample precipitation steps have been employed, the sample solution must contain certain components to ensure complete solubilization and denaturation prior to first-dimension IEF. These always include urea and one or more detergents. The lysis solution in the Appendix, solution A, containing urea and the zwitterionic detergent CHAPS, has been found to be effective for solubilizing a wide range of samples. Reductant and IPG Buffer are also frequently added to the sample solution to enhance sample solubility.

IEF performed under denaturing conditions gives the highest resolution and the cleanest results. Urea, a neutral chaotrope, is used as the denaturant in the first dimension of 2-D electrophoresis. It is always included in the 2-D sample solution at a concentration of at least 8 M. Urea solubilizes and unfolds most proteins to their fully random conformation, with all ionizable groups exposed to solution. Recently, the use of thiourea in addition to urea has been found to further improve solubilization, particularly of membrane proteins [9,13,51–53].

A non-ionic or zwitterionic detergent is always included in the sample solution to ensure complete sample solubilization and to prevent aggregation through hydrophobic interactions. Originally, either of two similar non-ionic detergents, NP-40 or Triton X-100, were used [1,2]. Subsequent studies have demonstrated that the zwitterionic detergent CHAPS is often more effective [54]. Non-ionic or zwitterionic detergents are used in concentrations up to 4%.

When difficulties in achieving full sample solubilization are encountered, the anionic detergent SDS can be used as a solubilizing agent. SDS is a very effective protein solubilizer, but because it is charged and forms complexes

with proteins, it cannot be used as the sole detergent for solubilizing samples for 2-D electrophoresis. A widely used method for negating the interfering effect of SDS is dilution of the sample into a solution containing an excess of CHAPS, Triton X-100, or NP-40. The final concentration of SDS should be 0.25% or lower, and the ratio of the excess detergent to SDS should be at least 8:1 [24,31,55].

Reducing agents are frequently included in the sample solution to break any disulphide bonds present and to maintain all proteins in their fully reduced state. The most commonly used reductant is dithiothreitol (DTT) at concentrations ranging from 20 to 100 mM. Dithioerythritol (DTE) is similar to DTT and can also be used as a reducing agent. Originally, 2-mercaptoethanol was used as a reductant, but higher concentrations of the reductant are required, and inherent impurities may result in artifacts [56]. More recently, the non-thiol reductant tributyl phosphine, at a concentration of 2 mM, has been used as a reductant for 2-D samples [57].

Carrier ampholytes or IPG Buffer [up to 2% (v/v)] can be included in the sample solution. They enhance protein solubility by minimizing protein aggregation due to charge-charge interactions.

A sample should remain in sample solution at room temperature for at least 1 hour for full denaturation and solubilization prior to centrifugation and subsequent sample application. Heating of the sample in the presence of detergent can aid in solubilization, but should only be done prior to the addition of urea, as heating in the presence of urea can introduce protein charge modifications. Sonication helps speed solubilization, particularly from material that is otherwise difficult to resuspend.

A widely used sample solution is the lysis solution given in the Appendix, solution A.

For a general review of protein solubilization for electrophoretic analysis, see [12].

Part II

First-dimension isoelectric focusing

3.0 First-dimension isoelectric focusing—overview

Amersham Pharmacia Biotech offers two different systems for the first-dimension separation: the Multiphor II system with associated accessories, and the IPGphor Isoelectric Focusing System. A comparison of these two systems is given in section 1.2.

A useful first-dimension separation requires selecting a first-dimension pH range appropriate for the sample as well as a suitable sample application method. Choice of immobilized pH gradient is discussed in section 3.2. Sample application methods and their selection are discussed in section 3.3.

The first-dimension separation procedure involves IPG strip rehydration, sample application, and isoelectric focusing. Preparation of the IPG strip rehydration solution is described in section 3.4. The protocols for IPG strip rehydration, sample application, and IEF are specific to the first-dimension system used and are described in section 3.5 for the Multiphor II system, and section 3.6 for the IPGphor Isoelectric Focusing System.

3.1 Background to isoelectric focusing (IEF)

IEF is an electrophoretic method that separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings (see Figure 7). The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl- termini. The isoelectric point is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. If the net charge of a protein is plotted versus the pH of its environment (see Figure 7), the resulting curve intersects the abscissa at the isoelectric point.

The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the *focusing* effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences.

The degree of resolution is determined by the slope of the pH gradient and the electric field strength. IEF is therefore performed at high voltages (typically in excess of 1,000 V). When the proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically below 1 mA). IEF of a given sample in a given electrophoresis system is generally performed for a constant number of volt-hours. (Volt-hours is the product of the voltage and the hours elapsed at that voltage.)

IEF performed under denaturing conditions gives the highest resolution and the cleanest results. Complete denaturation and solubilization achieved with a mixture of urea and detergent ensure that each protein is present in only one configuration and minimizes aggregation and intermolecular interaction.

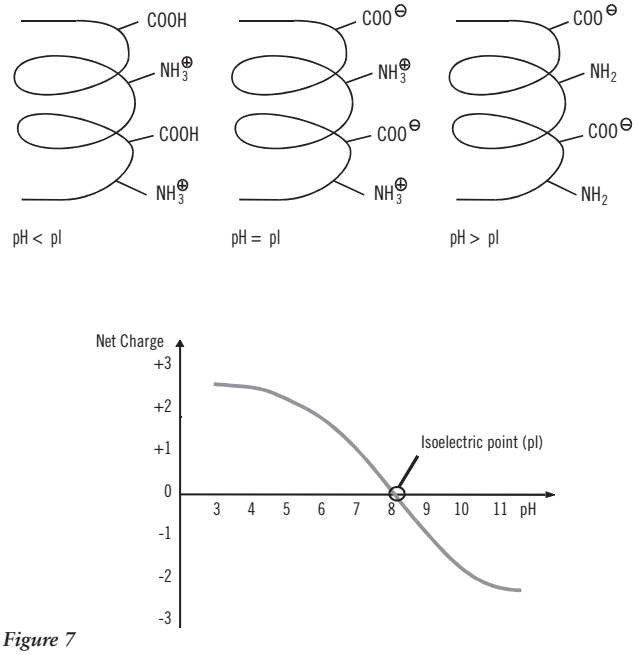


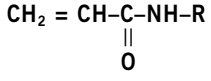
Figure 7

The original method for first-dimension IEF depended on carrier ampholyte-generated pH gradients in polyacrylamide tube gels [1,2]. Carrier ampholytes are small, soluble, amphoteric molecules with a high buffering capacity near their pI. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with pIs spanning a specific pH range. When a voltage is applied across a carrier ampholyte mixture, the carrier ampholytes with the lowest pI (and the most negative charge) move toward the anode, and the carrier ampholytes with the highest pI (and the most positive charge) move toward the cathode. The other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pHs. The result is a continuous pH gradient.

Although this basic method has been used in hundreds of 2-D electrophoresis studies, it has several limitations that have prevented its more widespread application:

- Carrier ampholytes are mixed polymers that are not well characterized and suffer from batch-to-batch manufacturing variations. These variations reduce the reproducibility of the first-dimension separation.
- Carrier ampholyte pH gradients are unstable and have a tendency to drift, usually toward the cathode, over time. Gradient drift adversely affects reproducibility by introducing a time variable. Gradient drift also causes a flattening of the pH gradient at each end, particularly above pH 9, rendering the 2-D technique less useful at pH extremes.
- The soft polyacrylamide tube gels have low mechanical stability. The gel rods may stretch or break, affecting reproducibility. Results are often dependent on the skill of the operator.

Because of the limitations of the carrier ampholytes method, an alternative technique for pH gradient formation was developed: immobilized pH gradients, or IPG. This technique was introduced by Bjellqvist and others in 1982 [58]. An immobilized pH gradient (IPG) is created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast. The buffers, called acrylamido buffers (Amersham Pharmacia Biotech Immobiline® reagents), are a set of well-characterized molecules, each with a single acidic or basic buffering group linked to an acrylamide monomer. Their general structure is the following:



R = weakly acidic or basic buffering group

Immobilized pH gradients are formed using two solutions, one containing a relatively acidic mixture of acrylamido buffers and the other containing a relatively basic mixture. The concentrations of the various buffers in the two solutions define the range and shape of the pH gradient produced. Both solutions contain acrylamide monomers and catalysts. During polymerization the acrylamide portion of the buffers copolymerize with the acrylamide and bisacrylamide monomers to form a polyacrylamide gel. Figure 8 is a graphical representation of the polyacrylamide matrix with attached buffering groups.

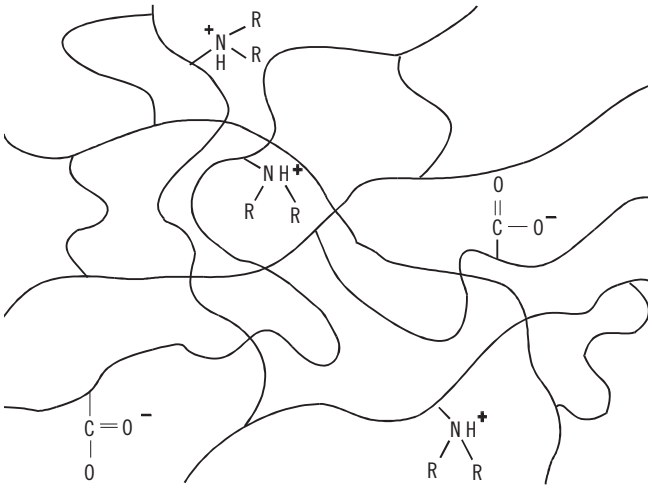


Figure 8

For improved performance and simplified handling, the IPG gel is cast onto a plastic backing. The gel is then washed to remove catalysts and unpolymerized monomers, which could otherwise modify proteins and interfere with separation. Finally the gel is dried and cut into 3-mm-wide strips. The resulting IPG strips can be rehydrated with a rehydration solution containing the necessary components for first-dimension IEF.

IEF is performed with the IPG strips placed horizontally on a flatbed electrophoresis unit. Advantages to using the flatbed format include the following:

- Isoelectric focusing requires efficient cooling for close temperature control, which can be effectively achieved on a horizontal ceramic cooling plate connected to a thermostatic circulator or a Peltier cooling plate.
- IEF requires high field strengths to obtain sharply focused bands, thus high voltages must be applied. A flatbed design is the most economical way to meet the necessary safety standards required to operate at such high voltages.

A. Görg *et al.* [3,4] pioneered the development and use of IPG strips for the first dimension of 2-D electrophoresis. The protocols presented in this manual are largely based on the work of A. Görg and her colleagues. The IPG strips are rehydrated in a solution containing the necessary additives and, optionally, the sample proteins. (The rehydration solution is described in detail in section 3.4.) IEF is performed by gradually increasing the voltage across the IPG strips to at least 3,500 V and maintaining this voltage for at least several thousand volt-hours. After IEF the IPG strips are equilibrated in equilibration solution and applied onto flatbed or vertical SDS-polyacrylamide gels.

When IPG strips are used for the first-dimension separation, the resultant 2-D maps are superior in terms of resolution and reproducibility. IPG strips are a marked improvement over the tube gels with carrier ampholyte-generated pH gradients:

- The first-dimension separation is more reproducible because the covalently fixed gradient cannot drift.
- The plastic-backed IPG strips are easy to handle. They can be picked up at either end with forceps or gloved fingers.
- The plastic support film prevents the gels from stretching or breaking.
- IPG technology increases the useful pH range on any single IPG strip; more very acidic and basic proteins can be separated.
- The IPG strips have a higher loading capacity for protein [59].
- The sample can be introduced into the IPG strip during rehydration [60,61].
- Precast Immobiline DryStrip gels are available from Amersham Pharmacia Biotech. These ready-made dry IPG strips eliminate the need to handle toxic acrylamide monomers, preparation time and effort are significantly reduced, and reproducibility of the pH gradient is assured.

3.2 Immobilized pH gradient selection

Ready-made IPG strips, Immobiline DryStrip gels, are available from Amersham Pharmacia Biotech with the pH gradients 4–7 L (linear), 6–11 L (linear), 3–10 L (linear), and 3–10 NL (non-linear). Available strip lengths are 7, 11, 13, and 18 cm. The pH 3–10 L IPG strips have a linear pH gradient between pH 3 and pH 10. The pH 3–10 NL IPG strips have a roughly sigmoidal gradient that gives improved resolution between pH 5 and pH 7.

If a specialized pH gradient is required, recipes for preparing custom narrow and wide immobilized pH gradients are given in [62].

A pH 3–10 IPG strip will display the widest range of proteins on a single 2-D gel. The narrower pH ranges are used for higher resolution separations in a particular pH range.

3.3 Sample application method selection

Sample can be applied either by including it in the rehydration solution or by applying it directly to the rehydrated IPG strip via sample cups or sample wells.

It is usually preferable to load the sample onto the IPG strip by including the sample in the rehydration solution (see section 3.4). Advantages to this mode of application include the following:

- This method allows larger quantities of protein to be loaded and separated [60,61].
- This method allows more dilute samples to be loaded.
- Because there is no discrete application point, this method eliminates the formation of precipitates at the application point that often occur when loading with sample cups.
- This method is technically simpler, avoiding problems of leakage that can occur when using sample cups.

There are, however, cases when one might prefer to load the sample following rehydration, immediately prior to IEF:

- If proteolysis or other protein modifications are a concern, overnight rehydration with sample may not be desired.
- Better results are often obtained on pH 6–11 L IPG strips when the sample is loaded anodically in a sample cup or sample well.

Guidelines for sample application after rehydration using the Multiphor II and Immobiline DryStrip Kit system are given in section 3.5.2.D. Sample is pipetted into sample cups precisely positioned on the surface of the IPG gels. Up to 100 µl per strip can be applied through the sample cups.

IPGphor system guidelines for sample application after rehydration are given in section 3.6.3. Sample is pipetted into sample application wells located at each end of the strip holder. Up to 7.5 µl of sample solution can be added to each side (i.e., 15 µl per well or 30 µl total if both sides of both wells are used).

3.4 IPG strip rehydration solution

IPG strips must be rehydrated prior to IEF. The IPG strips are rehydrated in the Immobiline DryStrip Reswelling Tray if the Multiphor II system is used for IEF, or in IPGphor strip holders, if the IPGphor is used.

Rehydration solution, which may or may not include the sample, is applied to the reservoir slots of the Reswelling Tray or the IPGphor strip holders, then the IPG strips are soaked individually. Rehydrated strips are 3 mm wide and approximately 0.5 mm thick.

3.4.1 Components of the rehydration solution

Selection of the optimal rehydration solution will depend on the specific protein solubility requirements of the sample. A typical solution generally contains urea, non-ionic or zwitterionic detergent, dithiothreitol (DTT), IPG Buffer (Amersham Pharmacia Biotech) appropriate to the pH range of the IPG strip, and dye. The sample may also be included. The role of each component is described below, as well as the recommended concentration range.

- **Urea** solubilizes and denatures proteins, unfolding them to expose internal ionizable amino acids. Commonly 8 M urea is used, but the concentration can be increased to 9 or 9.8 M if necessary for complete sample solubilization. It has recently been reported that using thiourea in addition to urea further improves solubilization, particularly of membrane proteins [9,13,51–53].
- **Detergent** solubilizes hydrophobic proteins and minimizes protein aggregation. The detergent must have zero net charge—use only non-ionic and zwitterionic detergents. CHAPS, Triton X-100, or NP-40 in a concentration of 0.5 to 4% are most commonly used.
- **Reductant** cleaves disulphide bonds to allow proteins to unfold completely. DTT or DTE (20 to 100 mM) is commonly used. 2-Mercaptoethanol can be used instead, but higher concentrations are required, and impurities may result in artifacts [56]. It has recently been reported that the non-thiol reductant tributyl phosphine can be used in first-dimension IEF [57]. Add the reductant just prior to use.
- **IPG Buffer (carrier ampholyte mixture)** can improve separations and sample solubility, particularly with high sample loads. IPG Buffers for each pH range are a mixture of carrier ampholytes that enhances sample solubility and produces more-uniform conductivity across the pH gradient during IEF without affecting the shape of the gradient. IPG Buffers are also specially formulated not to interfere with silver staining. Table 9 lists the recommended final concentration of IPG Buffer for the rehydration solution.

  The recommended IPG Buffer concentration for the IPGphor system is 0.5%, but up to 2% can be added if sample solubilization remains a problem.

  Note: Concentrations at the upper end of the recommended range may increase the time required for the voltage to reach its maximum setting during IEF, which can increase the time required for complete focusing.

  IPG Buffer can be included in the stock rehydration solution or added just prior to use. (IPG Buffer is included in the stock solution when multiple IPG strips of the same pH range will be used. IPG Buffer is added just prior to use to single aliquots of the stock solution when the same stock solution will be used with different pH range IPG strips.) See section 3.4.2.
- **Tracking dye** (bromophenol blue) provides a monitor for IEF progress at the beginning of the protocol. If the tracking dye does not migrate toward the anode, no current is flowing. Note, however, that the dye leaves the strip well before the sample is focused!
- **Sample** can be applied by including it in the rehydration solution. Up to 1 mg of sample per strip can be diluted into or redissolved in rehydration solution just prior to IEF. The amount of sample required is

TABLE 9. ADDITION OF IPG BUFFER TO THE REHYDRATION SOLUTION

IEF system	pH range of IPG strip	Suggested carrier ampholytes for rehydration solution	Recommended concentration
Multiphor II	4–7 L, 3–10 L or 3–10 NL	IPG Buffer with pH range identical to that of IPG strip	2% IPG Buffer (50 µl per 2.5 ml)
Multiphor II	6–11 L	pH 6–11 L IPG Buffer	0.5% IPG Buffer (12.5 µl per 2.5 ml)
IPGphor	4–7 L, 3–10 L, 3–10 NL, or 6–11 L	IPG Buffer with pH range identical to that of IPG strip	0.5% IPG Buffer (12.5 µl per 2.5 ml)

dictated in part by the detection or visualization method used. Radiolabeling requires a very small amount of sample, silver staining requires typically 1 to 100 µg of sample, and Coomassie blue staining and preparative applications require larger amounts.

3.4.2 Rehydration solution preparation

❶ Prepare the rehydration stock solution. Recommended formulations are listed in the Appendix, solutions B and C. (Select the formulation appropriate to the experiment.)

Note: Stock solution can be stored in 2.5 ml aliquots at –20 °C.

❷ Just prior to use, slowly thaw a 2.5 ml aliquot of stock solution. Add the appropriate amount of IPG Buffer, if it is not already included in the rehydration stock solution. (Refer to Table 9).

❸ Add 7 mg DTT and sample (if desired).

Note: DTT and the sample must be added fresh, just prior to use.

3.5 Multiphor II and Immobiline DryStrip Kit

3.5.1 IPG strip rehydration — Immobiline DryStrip Reswelling Tray

The Immobiline DryStrip Reswelling Tray has 12 independent reservoir slots that can each hold a single IPG strip up to 18 cm long. Separate slots allow the rehydration of individual IPG strips in a minimal volume of solution.

❶ Prepare the Reswelling Tray (Figure 9).

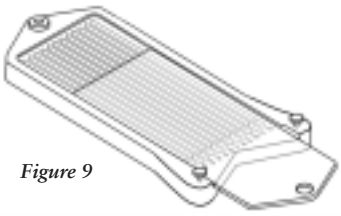


Figure 9

Slide the protective lid completely off the tray and level the tray by turning the leveling feet until the bubble in the spirit level is centred. Ensure that the tray is clean and dry.

❷ Apply the rehydration solution.

Pipette the appropriate volume of rehydration solution into each slot as indicated in Table 10. Deliver the solution slowly to the center of the slot. Remove any large bubbles.

Important: To ensure complete sample uptake, do not apply excess rehydration solution.

TABLE 10. REHYDRATION SOLUTION VOLUME PER IPG STRIP

IPG strip length (cm)	Total volume per strip[1] (µl)
7	125
11	200
13	250
18	350

[1] Including sample, if applied.

❸ Place the IPG strip (Figure 10).

Figure 10

Remove the protective cover from the IPG strip. Position the IPG strip with the gel side down and the pointed end of the strip against the sloped end of the slot. Lower the IPG strip onto the solution. To help coat the entire IPG strip, gently lift and lower the strip and slide it back and forth along the surface of the solution. Be careful not to trap bubbles under the IPG strip.

❹ Overlay the IPG strip with IPG Cover Fluid.

Overlay each IPG strip with 1.5 to 3 ml of IPG Cover Fluid to minimize evaporation and urea crystallization.

❺ Allow the IPG strip to rehydrate.

Slide the lid onto the Reswelling Tray and allow the IPG strips to rehydrate at room temperature. A minimum of 10 hours is required for rehydration; overnight is recommended. If the IPG strips swell unevenly, refer to Table 11.

❻ Prepare the Immobiline DryStrip Kit.

Before removing the IPG strips from the Reswelling Tray, prepare the Multiphor II Immobiline DryStrip Kit and the electrode strips as described in sections 3.5.2.A and 3.5.2.B.

TABLE 11. TROUBLESHOOTING IPG STRIP REHYDRATION IN RESWELLING TRAY

Symptom	Possible cause	Remedy
Uneven swelling of strips	*Note:* It is normal for the basic end to swell faster than the acidic end.	
	Dehydrated IPG strips were stored at or above room temperature for too long.	Do not allow dry IPG strips to sit at room temperature for longer than 10 minutes. Strips will pick up moisture from the air. Store IPG strips well sealed at temperatures below –20 °C.
	Incorrect volume of rehydration solution used.	Make sure the correct amount of solution is added to the slot in the Reswelling Tray.
	The rehydration time is too short.	Rehydrate the IPG strips for at least 10 hours.

3.5.2 Preparing for IEF

The components of the 2-D Immobiline DryStrip Kit include a tray and electrode holder, anode and cathode electrodes, a DryStrip aligner, a sample cup bar, and sample cups.

Procedures A and B below should be completed before the IPG strips are removed from the Reswelling Tray.

A. Prepare the Immobiline DryStrip Kit

❶ Clean all components of the Immobiline DryStrip Kit.

The Immobiline DryStrip tray, DryStrip aligner, electrodes, sample cup bar, and sample cups must be clean and ready for use. Clean with detergent, rinse thoroughly with distilled water, and allow to dry.

❷ Confirm electrical connections on Multiphor II.

Check that the red bridging cable in the Multiphor II unit is connected.

❸ Establish cooling.

Set the temperature on MultiTemp III Thermostatic Circulator to 20 °C.

Position the cooling plate on the Multiphor II unit and ensure that the surface is level.

Turn on MultiTemp III Thermostatic Circulator.

❹ Position the Immobiline DryStrip tray.

Pipette approximately 10 ml of IPG Cover Fluid onto the cooling plate. Position the Immobiline DryStrip tray on the cooling plate so the red (anodic) electrode connection of the tray is positioned at the top of the plate near the cooling tubes. Remove any large bubbles between the tray and the cooling plate; small bubbles can be ignored. The IPG Cover Fluid at this point serves to ensure good thermal contact between the cooling plate and the tray.

Connect the red and black electrode leads on the tray to the Multiphor II unit.

❺ Place the DryStrip aligner.

Pour about 15 ml of IPG Cover Fluid into the Immobiline DryStrip tray. Place the DryStrip aligner, 13-groove-side up, into the tray on top of the IPG Cover Fluid. The presence of air bubbles between the strip positions under the DryStrip aligner will not affect the experiment. Avoid getting IPG Cover Fluid on top of the aligner at this point, as it interferes with visualization of the grooves.

B. Prepare electrode strips

❶ Cut electrode strips to size.

Cut two IEF electrode strips to a length of 110 mm.

❷ Soak electrode strips with distilled water.

Place the electrode strips on a clean flat surface such as a glass plate. Soak each electrode strip with 0.5 ml distilled water. Blot with filter paper to remove excess water.

Important: Electrode strips must be damp, not wet. Excess water may cause streaking.

Note: Steps A and B above should be completed before proceeding.

C. Prepare for electrophoresis

❶ Remove the rehydrated IPG strip from the Reswelling Tray.

To remove an IPG strip from its slot in the Reswelling Tray, slide the tip of a pair of forceps along the sloped end of the slot and into the slight depression under the IPG strip. Grab the end of the strip with the forceps and lift the strip out of the tray.

❷ Rinse the IPG strip with deionized water.

Hold the IPG strip with the forceps and rinse briefly in a stream of deionized water delivered from a squeeze bottle. This rinse will remove excess rehydration solution and thus prevent formation of urea crystals on the gel surface during IEF. Place the IPG strip on its edge on a damp filter paper for several seconds to drain excess moisture. Avoid contact between the gel surface and the filter paper.

❸ Position the IPG strip in the DryStrip aligner (Fig. 11).

Figure 11

Immediately transfer the rehydrated IPG strips to adjacent grooves of the aligner in the Immobiline DryStrip tray. Place the strips with the pointed (acidic) end at the top of the tray near the red electrode (anode). The blunt end should be at the bottom of the tray near the black electrode (cathode). Align the IPG strips so that the anodic gel edges are lined up.

❹ Place the electrode strips.

Place the moistened electrode strips across the cathodic and anodic ends of the aligned IPG strips. The electrode strips must at least partially contact the gel surface of each IPG strip.

❺ Position the electrodes (Figure 12).

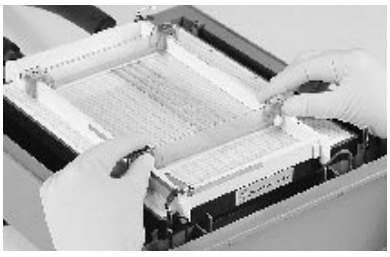


Figure 12

Each electrode has a side marked red (anode) or black (cathode). Align each electrode over an electrode strip, ensuring that the marked side corresponds to the side of the tray giving electrical contact. When the electrodes are properly aligned, press them down to contact the electrode strips. Check that the IPG strips are still aligned in their grooves.

D. Optional: Apply sample after gel rehydration

If the sample was not applied by means of the rehydration solution, it can be applied using the sample cups, immediately prior to isoelectric focusing. When sample cups are used, the sample load limits are lower and more specific. Guidelines on suitable sample loads for different gradients and IPG strips are given in Table 12. These values should be regarded as only a general guide. Suitable sample load will vary greatly among samples and with the sensitivity of the staining method used.

TABLE 12. SUITABLE SAMPLE LOADS WITH SAMPLE CUPS

Immobiline DryStrip		Suitable sample load (µg of protein)
7 cm	pH 4–7 L	4–8
7 cm	pH 6–11 L	8–16
7 cm	pH 3–10 L and pH 3–10 NL	2–4
11 cm	pH 4–7 L	10–20
11 cm	pH 6–11 L	20–40
11 cm	pH 3–10 L	4–8
13 cm	pH 4–7 L	15–30
13 cm	pH 6–11 L	30–60
13 cm	pH 3–10 L and pH 3–10 NL	8–15
18 cm	pH 4–7 L	30–60
18 cm	pH 6–11 L	60–120
18 cm	pH 3–10 L and pH 3–10 NL	15–30

❶ Prepare the sample.

Prepare the sample in a solution similar in composition to the rehydration solution used.

❷ Determine the point of sample application.

The optimal application point depends on the characteristics of the sample. When the proteins of interest have acidic pIs or when SDS has been used in sample preparation, sample application near the cathode is recommended. Anodic sample application is necessary with pH 6–11 gradients and preferred when pH 3–10 gradients are used. The optimal application point can vary with the nature of the sample. Empirical determination of the optimal application point is best.

❸ Position the sample cup bar.

Place sample cups on the sample cup bar, high enough on the bar to avoid touching the gel surface. Position the sample cup bar so that the sample cups are a few millimeters away from the cathodic or anodic electrode, depending on your sample. The sample cups must face the electrode. The sample cup bar has a spacer on one side. Slide the sample cup bar toward the anode/cathode until the spacer just touches the anodic/cathodic electrode.

❹ Press the sample cups against the IPG strips (Fig. 13).

Figure 13

Move the sample cups into position, one sample cup above each IPG strip, and press the sample cups down to ensure good contact with each IPG strip. This is perhaps the most critical part of the setup. Check that the strips are in their correct, straight position in the DryStrip aligner.

❺ Apply IPG Cover Fluid.

Once the sample cups are properly positioned, pour 70 to 80 ml of IPG Cover Fluid into the tray to completely cover the IPG strips. If the IPG Cover Fluid leaks into the sample cups correct the position of the sample cups, remove the fluid from the cups with a pipette, and check for leakage again. Add approximately 150 ml of additional IPG Cover Fluid to cover the sample cups. The IPG strips are submerged under a layer of IPG Cover Fluid to prevent drying of the IPG strip, precipitation of the components of the rehydration solution, and diffusion of gasses into the IPG strip.

❻ Apply the sample (Figure 14).

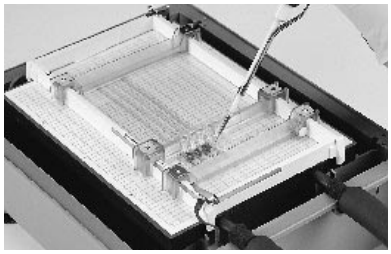


Figure 14

Apply sample (up to 100 µl per IPG strip) into the sample cups by pipetting under the surface of the IPG Cover Fluid. The sample should sink to the bottom of the cup. Watch for leakage.

Note: As mentioned in section 3.3, when sample is applied via sample cups, precipitates can form at the application point and the amount of protein that can be loaded is less than if the sample had been included in the rehydration solution. These limitations can sometimes be minimized with the following suggestions.

Protein precipitation and aggregation at the application point can sometimes be avoided:

- The sample should contain urea, non-ionic detergents, and IPG buffer or carrier ampholytes.
- Apply the sample in dilute solutions (60 to 100 µg protein per 100 µl).
- Limit the voltage to 10 to 30 V/cm for the initial 1 to 2 hours of focusing.
- Add Ultrodex™ resin to the sample [4].

For micropreparative applications, larger sample loads can be applied via sample cups:

- Load the sample cup repeatedly during IEF.
- Apply the sample at both the acidic and the basic ends (using two sample cup bars).

3.5.3 Isoelectric focusing guidelines

IEF in the Multiphor II system is conducted at very high voltages (up to 3,500 V) and very low currents (typically less than 1 mA) due to the low ionic strength within IPG strips. During IEF the current decreases while the voltage increases as proteins and other charged components migrate to their equilibrium positions. A typical IEF protocol generally proceeds through a series of voltage steps that begins at a relatively low value. Voltage is then gradually increased to the final desired focusing voltage, which is held for up to several hours. A low initial voltage minimizes sample aggregation and allows the parallel separation of samples with differing salt concentrations. A gradual increase in voltage is particularly advised for higher protein loads (100 µg or more per IPG strip).

Many factors affect the amount of time required for complete focusing, and each specific set of conditions (e.g., sample and rehydration solution composition, IPG strip length, and pH gradient) will require an empirical determination for optimal results. An approximate time is given in the example protocols provided in Table 13.

One factor that increases required focusing time is the presence of small ions, which must move to the ends of the IPG strips before protein focusing can occur. Larger quantities of protein also require more time to focus.

Note: Over-focusing is seldom a problem below 100,000 total volt-hours, but on longer runs it may contribute to horizontal streaking, visible in the 2-D result. (See also section 6.0, "Troubleshooting 2-D results.")

3.5.4 Protocol examples—Multiphor II

The protocols given in Table 13 are suitable for first-dimension isoelectric focusing of protein samples in typical analytical quantities with IPG Buffer concentrations of 0.5 to 2% in the rehydration solution. Optimal focusing time will vary with the nature of the sample, the amount of protein, and how the sample is applied.

For higher protein loads (up to 1 mg or more), the final focusing step of each protocol can be extended up to 100,000 volt-hours (Vh) if necessary.

Note: Sample application onto pH 6–11 L IPG strips by inclusion in the rehydration solution significantly prolongs the time required for complete focusing. Increase the recommended volt-hours in the final phase of the program by 6- to 10-fold for 7-cm-long IPG strips, 5- to 8-fold for 11-cm-long IPG strips, and 5- to 7-fold for 13- and 18- cm-long IPG strips.

3.5.5 Running a protocol

Ensure that the electrodes on the Immobiline DryStrip tray are connected and place the lid on the Multiphor II unit. Connect the leads on the lid to the power supply. Ensure that the current check on the EPS 3501 XL power supply is switched off. Begin IEF.

As isoelectric focusing proceeds, the bromophenol blue tracking dye migrates toward the anode. Note that the

TABLE 13. IMMOBILINE DRYSTRIP IEF GUIDELINES FOR MULTIPHOR II

Program EPS 3501 XL power supply in gradient mode with current check option turned off. IPG strip is rehydrated with a solution containing IPG Buffer of the corresponding pH range.

Immobiline DryStrip		Phase	Voltage (V)	Current (mA)	Power (W)	Duration (h:min)	Vh (recommended)
length	**pH range(s)**						
7 cm	*pH 4–7 L*	1	200	2	5	0:01	1
		2	3500[1]	2	5	1:30	2800
		3	3500	2	5	0:55–1:30	3200–5200
		Total				**2:25–3:00**	**6000–8000**
7 cm	*pH 6–11 L,[2] pH 3–10 L, and pH 3–10 NL*	1	200	2	5	0:01	1
		2	3500[1]	2	5	1:30	2800
		3	3500	2	5	0:35–1:05	2200–3700
		Total				**2:05–2:35**	**5000–6500**
11 cm	*pH 4–7 L*	1	300	2	5	0:01	1
		2	3500[1]	2	5	1:30	2900
		3	3500	2	5	2:20–3:30	8100–12100
		Total				**3:50–5:00**	**11000–15000**
11 cm	*pH 6–11 L,[2] and pH 3–10 L*	1	300	2	5	0:01	1
		2	3500[1]	2	5	1:30	2900
		3	3500	2	5	1:45–2:35	6100–9100
		Total				**3:15–4:05**	**9000–12000**
13 cm	*pH 4–7 L*	1	300	2	5	0:01	1
		2	3500[1]	2	5	1:30	2900
		3	3500	2	5	3:45–4:20	13100–18100
		Total				**5:15–5:50**	**16000–21000**
13 cm	*pH 6–11 L,[2] pH 3–10 L, and pH 3–10 NL*	1	300	2	5	0:01	1
		2	3500[1]	2	5	1:30	2900
		3	3500	2	5	3:10–4:00	11100–14100
		Total				**4:40–5:30**	**14000–17000**
18 cm	*pH 4–7 L*	1	500	2	5	0:01	1
		2	3500[1]	2	5	1:30	3000
		3	3500	2	5	5:40–7:40	20000–27000
		Total				**7:10–9:10**	**23000–30000**
18 cm	*pH 6–11 L,[2] pH 3–10 L, and pH 3–10 NL*	1	500	2	5	0:01	1
		2	3500[1]	2	5	1:30	3000
		3	3500	2	5	4:50–6:20	17000–22000
		Total				**6:20–7:50**	**20000–25000**

[1] During phase 2, the voltage will rise from the voltage set for phase 1 to 3500 V. The voltage will remain at 3500 V throughout phase 3.

[2] When applying sample onto pH 6–11 L IPG strips by inclusion in the rehydration, solution more time is required for complete focusing. Increase the recommended volt-hours (Vh) in the final phase of the program by 6– to 10–fold for 7-cm-long IPG strips, 5– to 8–fold for 11-cm-long IPG strips, and 5– to 7–fold for 13- and 18-cm-long IPG strips.

dye front leaves the IPG strip well before focusing is complete, so clearing of the dye is no indication that the sample is focused. If the dye does not migrate, no current is flowing. If this occurs, check the contact between the electrodes and the electrode strips.

After IEF proceed to the second-dimension separation immediately or store the IPG strips at –40 to –80 °C in screw-cap tubes. The 7 cm strips fit in disposable, 15 ml conical tubes; 11-, 13-, and 18 cm strips fit in 25 × 200 mm screw-cap culture tubes.

3.5.6 Troubleshooting

Table 14 lists possible problems that could be encountered during IEF and how to solve them.

TABLE 14. TROUBLESHOOTING FIRST-DIMENSION IEF: MULTIPHOR II AND IMMOBILINE DRYSTRIP KIT

Symptom	Possible cause	Remedy
Sample cups leak	Incorrect handling and placement of sample cups.	Sample cups are fragile and should not be taken on and off the application bar too many times. Make sure the sample cups are aligned with the IPG strips. Make sure the bottom of the sample cups are flat against the gel surface of the IPG strips. (See Figure 13.) *Note:* Leaks can often be detected prior to sample application: • Observe the IPG Cover Fluid when it is poured into the Immobiline DryStrip Kit tray. If it leaks in through the bottom of the sample cups, reposition the cups, remove the fluid with a pipette, and check for leakage again. • An optional check for leakage is to add 0.01% bromophenol blue dye solution to the cups. If the dye leaks out of a cup, the cup must be repositioned to eliminate the leak. *(Important:* The leak detection dye must be removed from the sample cup before loading the sample.)
Low current	This is normal for IPG gels. The gels have very low conductivity.	Usually, IPG IEF starts close to 1 mA and drops into the µA range. This depends on the number of IPG strips in the instrument.
	Power supply cannot detect the low µA range current and shuts off.	Make sure that the low-current shut-off has been bypassed (see power supply instructions). IPG IEF may start in a current range that is not detectable by the power supply.
	IPG Buffer omitted from rehydration solution.	Always include IPG Buffer in the rehydration solution.
No current at start of run	No electrode contact or lack of electrical continuity.	Check to make sure that all Multiphor II contacts are in place. Make sure that the metal band within the electrode contacts the metal band along the side of the Immobiline DryStrip tray. Note that the metal band within the electrode is only on the end marked with the red or black circle. Ensure that the bridging cable under the cooling plate is properly installed.
	IPG strip is improperly rehydrated.	Ensure that the IPG strip is rehydrated along its entire length.
	The high-voltage lead from the electrophoresis unit is not plugged into the power supply correctly.	Ensure that the plugs on the high-voltage leads fit securely into the output jacks on the power supply. Use the appropriate adapter if necessary.
Sample dye does not move out of the sample cup	It is normal for several hours to elapse before the sample dye leaves the sample cups.	
	The sample cups were pressed down so hard against the gel that they pushed through the gel to rest against the plastic backing. This blocks the current and physically prevents the protein from entering the IPG strip.	Replace IPG strip and reapply sample cup.
	The ionic strength of the sample is higher than that of the gel. As a result, the field strength in the sample zone is inadequate to move the protein out of the sample zone at an appreciable rate. Movement may stop all together.	Dilute the sample as much as possible or, just prior to loading, dialyse the sample to remove salts.
Sparking or burning of IPG strips	Conductivity of the sample/IPG strips is too high.	Ensure that the sample is adequately desalted. Or, before raising the voltage to maximum, include a prolonged low-voltage phase in the IEF protocol to allow the ions to move to the ends of the IPG strip.

3.6 IPGphor Isoelectric Focusing System

With the IPGphor Isoelectric Focusing System, both rehydration of the IPG strip and IEF occur in individual strip holders. Different-length holders are available for the different-length IPG strips. A strip holder is made of thermally conductive ceramic with built-in platinum electrodes and a transparent lid. The sample can be loaded by simply including it in the rehydration solution, or the sample can be loaded separately just prior to IEF. Once sample is applied to the IPG strip and the strip holder is in place on the IPGphor unit platform, the remaining steps are carried out automatically according to the chosen protocol. Up to 12 strip holders can be used.

3.6.1 IPG strip rehydration—IPGphor strip holder

❶ Prepare the strip holder(s).

Select the strip holder(s) corresponding to the IPG strip length chosen for the experiment.

Important: Handle the ceramic holders with care, as they are brittle.

Wash each holder with detergent to remove residual protein. Rinse thoroughly with double distilled water. Use a cotton swab or a lint-free tissue to dry the holder or allow it to air-dry. Handle clean holders with gloves to avoid contamination.

Note: The holder must be completely dry before use.

❷ Apply the rehydration solution (Figure 15).

Figure 15

Pipette the appropriate volume of rehydration solution into each holder as indicated in Table 15. Deliver the solution slowly at a central point in the strip holder channel away from the sample application wells. Remove any larger bubbles.

Important: To ensure complete sample uptake, do not apply excess rehydration solution.

TABLE 15. REHYDRATION SOLUTION VOLUME PER IPG STRIP

IPG strip length (cm)	Total volume per strip[1] (µl)
7	125
11	200
13	250
18	350

[1] Including sample, if applied.

❸ Place the IPG strip (Figures 16 and 17).

Figure 16

Figure 17

Remove the protective cover from the IPG strip. Position the IPG strip with the gel side down and the pointed (anodic) end of the strip directed toward the pointed end of the strip holder. Pointed end first, lower the IPG strip onto the solution. To help coat the entire strip, gently lift and lower the strip and slide it back and forth along the surface of the solution, tilting the strip holder slightly as needed to ensure complete and even wetting.

Finally, lower the cathodic (square) end of the IPG strip into the channel, making sure that the gel contacts the strip holder electrodes at each end. (The gel can be visually identified once the rehydration solution begins to dye the gel.) Be careful not to trap bubbles under the IPG strip.

❹ Apply IPG Cover Fluid.

Apply IPG Cover Fluid to minimize evaporation and urea crystallization. Pipette the fluid dropwise into one end of the strip holder until one-half of the IPG strip is covered. Then pipette the fluid dropwise into the other end of the strip holder, adding fluid until the entire IPG strip is covered.

❺ Place the cover on the strip holder.

Pressure blocks on the underside of the cover ensure that the IPG strip maintains good contact with the electrodes as the gel swells.

❻ Allow the IPG strip to rehydrate.

Rehydration can proceed on the bench top or on the IPGphor unit platform. Ensure that the holder is on a level surface. A minimum of 10 hours is required for rehydration; overnight is recommended. The rehydration period can be programmed as the first step of an IPGphor protocol. This is especially convenient if temperature control during rehydration is a concern.

3.6.2 Optional: Apply electrode pads

Under certain conditions, such as prolonged focusing, water may migrate toward one end of the IPG strip, causing the other end to begin drying out. This effect can

be minimized by placing paper electrode pads between the IPG strip and each strip holder electrode just before IEF. Electrode pads may also absorb ions that would otherwise accumulate at the ends of the IPG strip and possibly interfere with the separation.

❶ Prepare electrode pads.

Cut two 3-mm-wide electrode pads from a paper IEF electrode strip. Place on a clean, flat surface such as a glass plate and soak with deionized water. Remove excess water by blotting with filter paper.

Important: Electrode pads must be damp, not wet.

❷ Position electrode pads.

Remove cover from strip holder. Using forceps or tweezers, lift one end of the rehydrated IPG strip. Position an electrode pad over the electrode, then lower the IPG strip back into place. Repeat at the other end. Replace cover on strip holder.

3.6.3 Optional: Apply sample after gel rehydration

If the sample was not applied as a part of the rehydration solution, it can be applied immediately prior to IEF.

❶ Prepare sample.

Prepare the sample in a solution similar in composition to the rehydration solution used.

❷ Apply sample (Figure 18).

Figure 18

Remove cover from strip holder. Pipette the sample into either or both of the lateral wells at either end of the strip holder. Introduce the sample below the IPG Cover Fluid.

Up to 7.5 µl of sample solution can be added to each side (i.e., 15 µl per well or 30 µl total if both sides of both wells are used).

Note: The IPG strip backing is impermeable; do not apply the sample to the back of the strip.

Replace cover on strip holder.

3.6.4 Isoelectric focusing guidelines

IEF in the IPGphor system is conducted at very high voltages (up to 8,000 V) and very low currents (typically less than 50 µA per IPG strip) due to the low ionic strength within IPG strips. During IEF the current decreases while the voltage increases as proteins and other charged components migrate to their equilibrium positions. A typical IEF protocol generally proceeds through a series of voltage steps that begins at a relatively low value. Voltage is gradually increased to the final desired focusing voltage, which is held for up to several hours. A low initial voltage minimizes sample aggregation and allows the parallel separation of samples with differing salt concentrations. A gradual increase in voltage is particularly advised for higher protein loads (100 µg or more per IPG strip).

Many factors affect the amount of time required for complete focusing, and each specific set of conditions (e.g., sample and rehydration solution composition, IPG strip length, and pH gradient) requires an empirical determination for optimal results. An approximate time is given in the example protocols provided in Table 16. Factors that increase the required focusing time include residual ions, which must move to the ends of the IPG strips before protein focusing can occur; and the presence of IPG Buffers, which contribute to the ionic strength of the electrophoresis medium. A higher IPG Buffer concentration increases the conductivity of the IPG strip, resulting in a lower final voltage when the system is limited by the maximum current setting. Longer focusing times may therefore be required at IPG Buffer concentrations higher than 0.5%.

Results for larger quantities of protein (50 µg to in excess of 1 mg) and for samples loaded through sample application wells can be improved by an extended focusing time and a more gradual ramping to the maximum voltage.

Note: Complete focusing requires considerably more time with pH 6–11 L IPG strips than with the other pH gradients.

Note: It is generally preferable to program a protocol on the basis of volt-hours rather than time. At limiting current, the actual maximum voltage attainable and the speed at which it is attained can vary depending on the conductivity of the sample and other components of the rehydration solution. Because the optimal time for focusing can vary, programming the protocol based on volt-hours is preferred because it compensates for this variability.

Note: Exceeding the current limit of 50 µA per IPG strip is not recommended, as this may result in excessive heat generation and may damage the IPG strip and/or strip holder. Under extreme circumstances, the IPG strip may burn.

Note: Over-focusing is seldom a problem below 100,000 total volt-hours, but on longer runs it may contribute to

TABLE 16. IMMOBILINE DRYSTRIP IEF GUIDELINES FOR IPGPHOR ISOELECTRIC FOCUSING SYSTEM

50 µA per IPG strip
20 °C for both rehydration and IEF

pH gradients 4–7 L, 3–10 L, and 3–10 NL

	Step	Voltage	Step duration (h:min)	Volt-hours (Vh)	Gradient type
7 cm	1	rehydration	12:00[1]		
	2	500	0:30	250	Step-n-hold
	3	1000	0:30	500	Step-n-hold
	4	8000[2]	1:00	8000	Step-n-hold
11 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	2:00	16000	Step-n-hold
13 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	2:00	16000	Step-n-hold
18 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	4:00	32000	Step-n-hold

pH gradient 6–11 L

	Step	Voltage	Step duration (h:min)	Volt-hours (Vh)	Gradient type
7 cm	1	rehydration	12:00[1]		
	2	500	0:30	250	Step-n-hold
	3	1000	0:30	500	Step-n-hold
	4	8000[2]	3:45	30000	Step-n-hold
11 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	7:30	60000	Step-n-hold
13 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	9:30	75000	Step-n-hold
18 cm	1	rehydration	12:00[1]		
	2	500	1:00	500	Step-n-hold
	3	1000	1:00	1000	Step-n-hold
	4	8000[2]	12:30	100000	Step-n-hold
	5	8000[2]	2:30	20000	Step-n-hold

[1] The total rehydration time can be adjusted somewhat for convenience, but must be greater than 10 hours.

[2] This voltage may not be reached within the suggested step duration.

horizontal streaking, visible in the 2-D result. (See also section 6.0, "Troubleshooting 2-D results.")

3.6.5 Protocol examples—IPGphor

The protocols given in Table 16 are suitable for first-dimension isoelectric focusing of protein samples suspended in rehydration solution in typical analytical quantities (1 to 50 µg). The protocols are optimized for a rehydration solution containing 0.5% IPG Buffer. The recommended current limit is 50 µA per IPG strip. Recommended focusing times are given, but the optimal length of time will depend on the nature of the sample, the amount of protein, and the method of sample application. Please refer to the IPGphor User Manual for instructions on how to program a protocol.

3.6.6 Running a protocol

Ensure that the strip holders are properly positioned on the IPGphor platform. (Use the guidemarks along the sides of the platform to position each strip holder and check that the pointed end of the strip holder is over the anode [pointing to the back of the unit] and the blunt end is over the cathode. Please refer to the IPGphor User Manual.) Check that both external electrode contacts on the underside of each strip holder make metal-to-metal contact with the platform.

Close the safety lid. At least two of the three pressure pads under the safety lid must press gently against the cover of each strip holder to ensure contact between the electrodes and the electrode areas. Begin IEF.

As isoelectric focusing proceeds, the bromophenol blue tracking dye migrates toward the anode. Note that the dye front leaves the IPG strip well before focusing is complete, so clearing of the dye is no indication that the sample is focused. If the dye does not migrate, no current is flowing. If this occurs, check the contact between the external face of the strip holder electrodes and the electrode areas on the instrument and between the rehydrated gel and the internal face of the electrodes.

Note: It is possible that the programmed maximum voltage will not be reached with the shorter IPG strips or with samples with high conductivity.

After IEF proceed to the second-dimension separation immediately or store the IPG strips at –40 to –80 °C in screw-cap tubes. The 7 cm strips fit in disposable, 15 ml conical tubes; 11-, 13-, 18 cm strips fit in 25 × 200 mm screw-cap culture tubes.

3.6.7 Troubleshooting

Table 17 lists possible problems that could be encountered during IEF and how to solve them.

TABLE 17. TROUBLESHOOTING FIRST-DIMENSION IEF: IPGPHOR

Symptom	Possible cause	Remedy
Current too low or zero	Electrical continuity is impeded.	Check the external electrode contacts: The electrodes at the bottom of the strip holder (one at each end) must make metal-to-metal contact with the appropriate electrode contact area. Check the internal electrode contacts: The gel (which becomes visible because of the dye in the rehydration solution) must contact *both* electrodes in the strip holder. Check that the IPG strip is fully rehydrated along its entire length. Electrical contact at the electrodes is reduced by incomplete rehydration.
Voltage too low or does not reach the maximum set value	The IPGphor protocol settings are incorrect for the experiment.	Check that the current limit is properly set. Check that the actual number of strips on the IPGphor platform equals the number of strips entered in the protocol.
	Conductivity/ionic strength is too high.	Prepare the sample to yield a salt concentration less than 10 mM. The recommended IPG Buffer concentration is 0.5%. A maximum of 2% is advisable only if sample solubility is a problem.
Sparking or burning in the strips	Current limit setting is too high.	Do not exceed the maximum recommended setting of 50 µA per IPG strip.
	The IPG strip is not fully rehydrated.	Ensure that the IPG strips are rehydrated with a sufficient volume of rehydration solution. Remove any large bubbles trapped under the IPG strip after placing on rehydration solution. Check that the entire IPG strip surface is wetted.
	The IPG strip dried during IEF.	Always apply IPG Cover Fluid to prevent dehydration of a rehydrated IPG strip.

Part III

Second-dimension SDS-PAGE

4.0 Second-dimension SDS-PAGE—overview

After IEF the second-dimension separation can be performed on various flatbed or vertical systems, depending on factors such as those discussed in section 1.2, 'Equipment choices.' SDS-PAGE consists of four steps: (1) preparing the second-dimension gel, (2) equilibrating the IPG strip(s) in SDS buffer, (3) placing the equilibrated IPG strip on the SDS gel, and (4) electrophoresis.

In this guide the equilibration step is described first because it is a protocol common to both vertical and flatbed systems. Gel preparation, IPG strip placement, and electrophoresis protocols, on the other hand, are specific to the orientation of the gel. Sections 4.3 and 4.4 describe these protocols as they apply to vertical systems and Multiphor II flatbed systems, respectively. Note however, that the second-dimension gel must be prepared before the equilibration step is started.

4.1 Background to SDS-PAGE

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) is an electrophoretic method for separating polypeptides according to their molecular weights (MW). The technique is performed in polyacrylamide gels containing sodium dodecyl sulphate (SDS). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 grams SDS per gram protein. The bound SDS masks the charge of the proteins themselves, forming anionic complexes with constant net negative charge per unit mass. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures. The proteins are totally unfolded when a reducing agent such as DTT is employed. The disulphide bonds, which can form between cysteine residues, are cleaved, and the polypeptides become flexible rods of negative charges with equal "charge densities," or charge per unit length. When proteins are treated with both SDS and a reducing agent, separations exclusively by molecular weight are possible. In fact, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle. (*Note:* This linear relationship is valid only for a certain molecular weight range that is determined by the polyacrylamide percentage.)

The most commonly used buffer system for second-dimension SDS-PAGE is the tris-glycine system described by Laemmli [63]. Other buffer systems can be used, particularly the tris-tricine system of Schägger and von Jagow [64] for resolution of polypeptides in the size range below 10 kDa. ExcelGel precast gels for second-dimension SDS-PAGE on the Multiphor II flatbed system utilize a different tris-tricine buffer system.

4.2 IPG strip equilibration

The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye. An additional optional equilibration step replaces the reductant with iodoacetamide.

4.2.1 Equilibration solution components

Equilibration introduces reagents essential for the second-dimension separation.

- **Equilibration buffer** (50 mM Tris-HCl, pH 8.8) maintains IPG strip pH in a range appropriate for electrophoresis.
- **Urea** (6 M), together with glycerol, reduces the effects of electroendosmosis by increasing the viscosity of the buffer [3]. Electroendosmosis is due to the presence of fixed charges on the IPG strip in the electric field and can interfere with protein transfer from the IPG strip to the second-dimension gel.
- **Glycerol** (30%), together with urea, reduces electroendosmosis and improves transfer of protein from the first to the second dimension [3].
- **DTT** preserves the fully reduced state of denatured, unalkylated proteins.
- **Sodium dodecyl sulphate (SDS)** denatures proteins and forms negatively charged protein-SDS complexes. The amount of SDS bound to a protein, and therefore the additional negative charge, is directly proportional to the mass of the protein. Thus, electrophoresis of proteins through a sieving gel in the presence of SDS separates proteins on the basis of molecular mass.

- **Iodoacetamide** alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis. Protein reoxidation during electrophoresis can result in streaking and other artifacts. Iodoacetamide also alkylates residual DTT to prevent point streaking and other silver-staining artifacts [65]. Iodoacetamide is introduced in a second equilibration step. This step is optional when SDS-PAGE is performed in a vertical second-dimension system, but required when SDS-PAGE is performed on a flatbed second-dimension system, especially when the flatbed separation is to be visualized by silver staining. Equilibration with iodoacetamide is also used to minimize unwanted reactions of cysteine residues (i.e., when mass spectroscopy is to be performed on the separated proteins).
- **Tracking dye** (bromophenol blue) allows monitoring of electrophoresis.

4.2.2 Equilibration steps

Note: The second-dimension gel must be ready for use prior to IPG strip equilibration. See sections 4.3.1 and 4.4.1 for preparation of vertical and horizontal gels, respectively.

❶ Prepare equilibration solution.

Prepare SDS equilibration buffer (see Appendix, solution D). This is a stock solution. Just prior to use, add 100 mg DTT per 10 ml SDS equilibration buffer.

❷ Equilibration.

Place the IPG strips in individual tubes with the support film toward the tube wall (screw-cap culture tubes work well). Add DTT-containing equilibration solution to each tube. Suggested volumes are 10 ml for 18 cm IPG strips, 5–10 ml for 11 cm or 13 cm IPG strips, and 2.5–5 ml for 7 cm IPG strips. Cap the tube or seal it with flexible paraffin film and place it on its side on a rocker. Equilibrate for 15 minutes.

❸ Second equilibration (recommended for flatbed second dimension, optional for vertical second dimension).

A second equilibration may be performed with an iodoacetamide solution (without DTT). Prepare a solution of 250 mg iodoacetamide per 10 ml SDS equilibration buffer.

Note: This second equilibration step reduces point streaking and other artifacts when using a flatbed system for the second dimension.

Decant the first equilibration solution and add iodoacetamide-containing equilibration solution to each tube. Suggested volumes are 10 ml for 18 cm IPG strips, 5–10 ml for 11 cm or 13 cm IPG strips, and 2.5–5 ml for 7 cm IPG strips. Cap the tube or seal it with flexible paraffin film, place it on its side on a rocker, and equilibrate for 15 minutes.

❹ Drain moisture from IPG strips (flatbed second dimension only).

After equilibration place the IPG strips on filter paper moistened with deionized water. To help drain the equilibration solution, place the IPG strips so that they rest on an edge. IPG strips can be left in this position for up to 10 minutes without noticeably affecting the spot sharpness. Alternatively, the IPG strips can be gently blotted with moistened filter paper to remove excess equilibration buffer.

4.3 Vertical systems

4.3.1 Preparing SDS slab gels—vertical systems

The instructions provided below for the preparation of vertical SDS-polyacrylamide gels employ the tris-glycine system of Laemmli [63]. Vertical second-dimension gels are most conveniently cast several at a time, in a multiple gel caster (see 'Ordering information'). For assembly of the gel cassette, refer to the relevant User Manual.

❶ Select the gel percentage.

a. Single percentage gel versus gradient gel.

Single percentage gels offer better resolution for a particular MW window. A commonly used second-dimension gel for 2-D electrophoresis is a homogeneous gel containing 12.5% total acrylamide.

When a gradient gel is used, the overall separation interval is wider and the linear separation interval is larger. In addition, bands are sharper because the decreasing pore size functions to minimize diffusion. A gradient gel requires more skill to cast, however. For detailed instructions on gradient preparation, see the User Manual for the relevant gel unit gradient maker and gel caster.

Note: Stacking gels are not necessary for vertical 2-D gels.

b. Whether single percentage or gradient, the appropriate percentage gel is selected according to the range of separation desired (see Table 18).

TABLE 18. RECOMMENDED ACRYLAMIDE CONCENTRATIONS FOR PROTEIN SEPARATION

% Acrylamide in resolving gel	Separation Size Range (MW × 10^{-3})
Single percentage:	
5%	36–200
7.5%	24–200
10%	14–200
12.5%	14–100[1]
15%	14–60[1]
Gradient:	
5–15%	14–200
5–20%	10–200
10–20%	10–150

[1]The larger proteins fail to move significantly into the gel.

❷ Select the gel thickness.

Either 1.0- or 1.5-mm-thick spacers can be used for all vertical formats. Thinner gels stain and destain more quickly and generally give less background staining. Thicker gels allow easier positioning of the IPG strip on the surface of the SDS gel and have a higher protein capacity. Thicker gels are also less fragile and easier to handle.

❸ Prepare the gel solution.

a. The total volume of solution needed depends on the gel size, the gel thickness, and the number of gels cast. Table 19 gives volumes of gel solution required per gel for the various possible vertical gel formats.

TABLE 19. VOLUMES REQUIRED PER VERTICAL GEL

Casting system	Volume (ml)
Hoefer® miniVE or SE 260 (10 × 10.5 cm plates)	
1-mm-thick spacers	10
1.5-mm-thick spacers	15
Hoefer® SE 600 (18 x 16 cm plates)	
2-cm-wide × 1-mm-thick spacers	30
2-cm-wide × 1.5-mm-thick spacers	40
1-cm-wide × 1-mm-thick spacers	30
1-cm-wide × 1.5-mm-thick spacers	45
Hoefer® DALT	see User Manual

b. Calculate the formulation of the gel solution. The recipes given in Table 20 produce 100 ml of solution for a single percentage gel. The recipes in Table 21 produce 50 ml each of light and heavy solution for a gradient gel. These recipes are to be scaled up or down, depending on the volume required.

c. Prepare the gel solution in a vacuum flask, omitting the TEMED and ammonium persulphate. Add a small magnetic stir bar.

Stopper the flask and apply a vacuum for several minutes while stirring on a magnetic stirrer.

Add the TEMED and ammonium persulphate and gently swirl the flask to mix, being careful not to generate bubbles. Immediately pour the gel.

❹ Pour and prepare the gel.

Fill the gel cassette to 3 to 10 mm below the top. (No stacking gel layer is required.)

Overlay each gel with a thin layer (100 to 500 µl) of water-saturated *n-*, *i-*, or *t*-butanol immediately after pouring to minimize gel exposure to oxygen and to create a flat gel surface.

After allowing a minimum of 1 hour for polymerization, inspect each gel and ensure that polymerization is even and complete and that the top surface of each gel is straight and flat. Remove the overlay and rinse the gel surface with gel storage solution (see Appendix, solution I).

❺ Storage of unused gels.

Gels not used immediately can be stored for future use at 4 °C for up to two weeks. Gel storage solution (see Appendix, solution I) is pipetted over the top gel surface, and the gel cassette is sealed with flexible paraffin film. Alternatively, the gel cassettes can be stored fully immersed in gel storage solution.

Note: For further information on the preparation of second-dimension vertical SDS slab gels, refer to the User Manuals for the respective vertical gel unit and gel caster.

TABLE 20. RECIPES FOR SINGLE PERCENTAGE GELS

(Preparation of stock solutions is described in the Appendix—solutions E, F, G, and H.)

Final gel concentration	**5%**	**7.5%**	**10%**	**12.5%**	**15%**
Monomer stock solution (solution E)	16.7 ml	25 ml	33.3 ml	41.7 ml	50 ml
4X Resolving gel buffer (solution F)	25 ml	25 ml	25 ml	25 ml	25 ml
10% SDS (solution G)	1 ml	1 ml	1 ml	1 ml	1 ml
Double distilled water	56.8 ml	48.5 ml	40.2 ml	31.8 ml	23.5 ml
10% Ammonium persulphate* (solution H)	500 µl	500 µl	500 µl	500 µl	500 µl
TEMED*	33 µl	33 µl	33 µl	33 µl	33 µl
Total volume	**100 ml**	**100 ml**	**100 ml**	**100 ml**	**100 ml**

*Add after deaeration.

TABLE 21. RECIPES FOR GRADIENT GELS

(Preparation of stock solutions is described in the Appendix—Solutions E, F, G, and H.)

Light solution—final concentration	**5%**	**7.5%**	**10%**	**12.5%**	**15%**
Monomer stock solution (solution E)	8.4 ml	12.5 ml	16.7 ml	21.0 ml	25 ml
4X Resolving gel buffer (solution F)	12.5 ml	12.5 ml	12.5 ml	12.5 ml	12.5 ml
10% SDS (solution G)	500 µl	500 µl	500 µl	500 µl	500 µl
Double distilled water	28.5 ml	24.5 ml	20.1 ml	16.0 ml	12.0 ml
10% Ammonium persulphate* (solution H)	165 µl	165 µl	165 µl	165 µl	165 µl
TEMED*	16.5 µl	16.5 µl	16.5 µl	16.5 µl	16.5 µl
Total volume	**50 ml**	**50 ml**	**50 ml**	**50 ml**	**50 ml**
Heavy solution—final concentration	**10%**	**12.5%**	**15%**	**17.5%**	**20%**
Monomer stock solution (solution E)	16.7 ml	21.0 ml	25.0 ml	29.2 ml	33.3 ml
4X Resolving gel buffer (solution F)	12.5 ml	12.5 ml	12.5 ml	12.5 ml	12.5 ml
Sucrose	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g
10% SDS (solution G)	500 µl	500 µl	500 µl	500 µl	500 µl
Double distilled water	16.2 ml	11.7 ml	7.7 ml	3.5 ml	0 ml
10% Ammonium persulphate* (solution H)	165 µl	165 µl	165 µl	165 µl	165 µl
TEMED*	16.5 µl	16.5 µl	16.5 µl	16.5 µl	16.5 µl
Total volume	**50 ml**	**50 ml**	**50 ml**	**50 ml**	**50 ml**

*Add after deaeration.

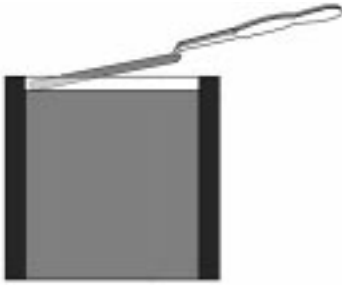


Figure 19

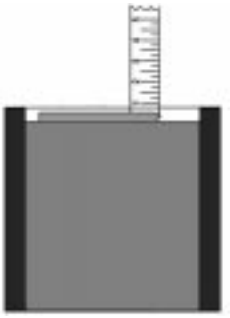


Figure 20

Figure 21

4.3.2 Applying the equilibrated IPG strip

(See section 4.2.2 for equilibration protocol.)

❶ Place the IPG strip.

Dip the IPG strip (from section 4.2.2) in the SDS electrophoresis buffer (see Appendix, solution J) to lubricate it. Position the IPG strip between the plates on the surface of the second-dimension gel with the plastic backing against one of the glass plates (Figure 19). With a thin plastic ruler, gently push the IPG strip down so the entire lower edge of the IPG strip is in contact with the top surface of the slab gel (Figure 20). Ensure that no air bubbles are trapped between the IPG strip and the slab gel surface or between the gel backing and the glass plate.

❷ Optional: Apply molecular weight marker proteins.

The markers are applied to a paper IEF sample application piece in a volume of 15 to 20 µl. For less volume, cut the sample application piece proportionally. Place the IEF application piece on a glass plate and pipette the marker solution onto it, then pick up the application piece with forceps and apply to the top surface of the gel next to one end of the IPG strip. The markers should contain 200 to 1,000 ng of each component for Coomassie staining and about 10 to 50 ng of each component for silver staining.

❸ Seal the IPG strip in place.

Imbedding the IPG strip in agarose prevents it from moving or floating in the electrophoresis buffer.

Prepare agarose sealing solution (see Appendix, solution K).

Melt each aliquot as needed in a 100 °C heat block (each gel will require 1 to 1.5 ml). It takes approximately 10 minutes to fully melt the agarose. (*Tip:* An ideal time to carry out this step is during IPG strip equilibration.) Allow the agarose to cool to 40 to 50 °C and then slowly pipette the amount required to seal the IPG strip in place (Figure 21). Pipetting slowly avoids introducing bubbles. Allow a minimum of 1 minute for the agarose to cool and solidify.

❹ Finish assembling the electrophoresis unit and add SDS electrophoresis buffer. (See Appendix, solution J.)

4.3.3 Electrophoresis conditions

Table 22 lists the recommended conditions for the Hoefer miniVE, SE 260, and SE 600. For Hoefer DALT conditions, please see the User Manual. Electrophoresis is performed at constant current in two steps. During the initial migration and stacking period, the current is approximately half of the value required for the separation. Stop electrophoresis when the dye front is approximately 1 mm from the bottom of the gel.

Cooling is optional; however, temperature control improves gel-to-gel reproducibility, especially if the ambient temperature of the laboratory fluctuates significantly. Do not cool SDS gels below 15 °C.

After electrophoresis, remove gels from their gel cassettes in preparation for staining or blotting. Notch or mark each gel at the upper corner nearest the pointed end of the IPG strip to identify the acidic end of the first-dimension separation.

TABLE 22. RECOMMENDED ELECTROPHORESIS CONDITIONS FOR SECOND-DIMENSION VERTICAL GELS

	Step	Current (mA/gel)	Duration (h:min)
Hoefer miniVE or SE 260			
1.5-mm-thick gels	1	15	0:15
	2	30	1:30[1]
1.0-mm-thick gels	1	10	0:15
	2	20	1:30[1]
Hoefer SE 600			
1.5-mm-thick gels	1	15	0:15
	2	30[2]	5:00[1]
1.0-mm-thick gels	1	10	0:15
	2	20[2]	5:00[1]

[1] The time shown is approximate. Stop electrophoresis when the dye front is 1 mm from the bottom of the gel.

[2] Currents up to 50% higher may be used if only two gels per unit are being run (no divider plates) and the unit is being cooled with a thermostatic circulator.

4.3.4 Troubleshooting

Table 23 lists possible problems that could be encountered during vertical SDS-PAGE and how to solve them.

TABLE 23. TROUBLESHOOTING VERTICAL SECOND-DIMENSION SDS-PAGE

Symptom	Possible cause	Remedy
No current at start of run	Insufficient volume of buffer in upper or lower reservoir.	Ensure that both reservoirs contain enough SDS electrophoresis buffer to contact both upper and lower electrode wires. Check for leaks.
The second dimension separation proceeds too slowly	SDS electrophoresis buffer is prepared incorrectly, or resolving gel buffer is prepared incorrectly.	Make fresh solutions.
	Acrylamide solution is too old.	Prepare fresh monomer stock solution.
Dye front curves up (smiles) at the edges	Gel is not properly cooled.	During electrophoresis, actively cool gel using a thermostatic circulator. Use the maximum possible volume of buffer in the lower reservoir.
	Current is too high.	Limit current to values suggested in Table 22.
Dye front curves down (frowns)	Gel is poorly polymerized near the spacers.	Degas the gel solution or increase the amount of ammonium persulphate and TEMED by 50%.
	Improper instrument assembly (SE 600).	Ensure that the gasket is not pinched.
	Leakage of upper reservoir.	Ensure that an adequate level of buffer is in the upper reservoir.
Dye front is irregular	Poor, uneven polymerization of gel.	Degas the gel solution or increase the amount of ammonium persulphate and TEMED by 50%.
	The top surface of the second-dimension gel is not flat.	Immediately after pouring the gel, overlay the surface with water-saturated butanol.

4.4 Multiphor II flatbed system

4.4.1 ExcelGel preparation

Two sizes of precast ExcelGel gradient SDS gels are available: The 110 × 250 mm gel contains an 8 to 18% acrylamide gradient, and the 180 × 250 mm gel contains a 12 to 14% acrylamide gradient. Either gel accepts a single 18- or 13 cm IPG strip, two 11 cm, or three 7 cm IPG strips. Placing shorter IPG strips end-to-end is ideal for comparative studies. For maximum resolution, the larger gel coupled with the 18 cm IPG strip is the best choice.

Important: A flatbed second-dimension system is not recommended if the first dimension has been run on a pH 6–11 IPG strip.

❶ Equilibrate the IPG strips.

Just prior to preparing the ExcelGel SDS gel, equilibrate the IPG strips as described in section 4.2.2.

❷ Prepare the Multiphor II unit.

Set the temperature on the MultiTemp III Thermostatic Circulator to 15 °C. Pipette 2.5 to 3.0 ml of IPG Cover Fluid or kerosene onto the Multiphor II cooling plate.

❸ Place the ExcelGel SDS gel.

Remove the gel from the foil package by cutting away the edges of the package. A notch at the lower-left corner of the film identifies the 18% or 14% (i.e., anodic) end.

Note: The gel is cast on a plastic support film and does not cover the film entirely.

Markings on the plastic cover of the gel indicate the direction of electrophoresis. Orient the gel according to these markings, remove the cover, and place the gel on the cooling plate.

Note: Avoid trapping bubbles between the gel and the cooling plate. Avoid getting IPG Cover Fluid or kerosene on the gel surface, as this may cause the buffer strips to slide during electrophoresis.

Separation quality is improved if the gel surface is allowed to dry, uncovered, for about 5 minutes before proceeding.

❹ Position the cathodic buffer strip (Figure 22).

Peel back the foil on the colorless cathodic (–) ExcelGel SDS buffer strip. Place the buffer strip with the smooth, narrow face downward along and in complete contact

with the cathodic (–) edge of the SDS gel. Avoid trapping air bubbles between the gel and the buffer strip. If the buffer strip breaks, piece it together on the gel.

Note: Vinyl gloves tend to stick less to the buffer strips than other types of plastic gloves. If sticking persists, dampen the gloves with distilled water or a 5% SDS solution.

❺ Position the anodic buffer strip.

Repeat step 4 with the yellow-colored anodic (+) ExcelGel buffer strip, placing it along and in contact with the anodic edge of the SDS gel.

4.4.2 Applying the equilibrated IPG strip

(See section 4.2.2 for the equilibration protocol.)

❶ Place the IPG strip(s) (Figure 23).

Once the equilibrated IPG strips (from section 4.2.2) have drained for at least 3 minutes, place the IPG strips, gel side down, on the SDS gel so that the cathodic buffer strip and the IPG strip are parallel to each other and 2 to 3 mm apart.

❷ Place sample application pieces (Figure 24).

Place one IEF sample application piece on the SDS gel, underneath the plastic tab formed by the overhanging gel support film at each end of the IPG strip(s). Be sure the application pieces are positioned so that they touch the ends of the IPG strip.

Note: Application pieces absorb water that flows out of the IPG strips during electrophoresis.

❸ Ensure contact between IPG strip and ExcelGel.

Make sure that the IPG strip is in full, direct contact with the SDS gel. To remove any bubbles, stroke the plastic backing of the IPG strip gently with a pair of forceps.

❹ Optional: Apply molecular weight marker proteins.

If loading marker proteins, place an extra application piece on the surface of the gel just beyond the end of the IPG strip. Pipette the markers onto the extra sample application piece. Apply the markers in a volume of 15 to 20 µl. For less volume, cut the sample application piece proportionally. The markers should contain 200 to 1,000 ng of each component for Coomassie staining and about 10 to 50 ng of each component for silver staining.

❺ Position electrodes (Figure 25).

Place the IEF electrode holder on the electrophoresis unit, in the upper position, and align the electrodes with the centre of the buffer strips. Plug in the electrode connectors and carefully lower the electrode holder onto the buffer strips. Check that the buffer strips have not moved.

4.4.3 Electrophoresis conditions

Place the safety lid on the Multiphor II. Connect the power supply. Recommended electrical settings and running times are listed in Table 24.

Note: It is important to use a protocol with a low-current sample entry phase. Remove the IPG strip and application pieces and move the cathodic buffer strip prior to the second, higher current phase (as indicated in footnote 1 of Table 24).

TABLE 24. ELECTROPHORESIS CONDITIONS FOR EXCELGEL

	Step	Voltage (V)	Current (mA)	Power (W)	Duration (h:min)
ExcelGel SDS, gradient, 8–18%	1	600	20	30	0:25–0:30[1]
	2	600	50	30	1:10[2]
ExcelGel XL SDS, gradient, 12–14%	1	1000	20	40	0:45[1]
	2	1000	40	40	2:40[2]

[1] When the bromophenol blue dye front has moved away from the IPG strip by 4–6 mm for ExcelGel XL SDS 12–14% or by 1–2 mm for ExcelGel SDS 8–18%, remove the IPG strip and the application pieces. Then move the cathodic buffer strip forward to cover the area of the removed IPG strip. Adjust the position of the cathodic electrode.

[2] Electrophoresis is stopped 5 minutes after the bromophenol blue front has just reached the anodic buffer strip. Remove and discard the buffer strips.

Figure 22

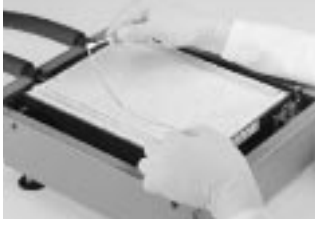


Figure 23

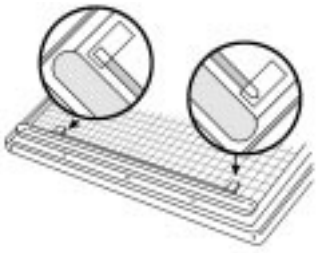


Figure 24

Figure 25

4.4.4 Troubleshooting

Table 25 lists possible problems that could be encountered during second-dimension SDS-PAGE using the Multiphor II flatbed system and how to solve them.

TABLE 25. TROUBLESHOOTING SECOND-DIMENSION SDS-PAGE: MULTIPHOR II FLATBED SYSTEM

Symptom	Possible cause	Remedy
No current at start of run.	The electrode cable is not plugged in.	Ensure that all cables are properly connected.
Dye front curves up (smiles) at one edge	Cathodic buffer strip does not contact the gel at the one edge.	Ensure that the cathodic buffer strip is centred and covers the entire width of the second-dimension gel.
Dye front curves up (smiles) at both edges	Inadequate cooling.	Ensure that the thermostatic circulator is connected to the Multiphor II unit and functioning correctly.
Dye front is irregular	Some dye front irregularity results from the use of IPG Buffer and does not affect results.	
	Buffer strips or ExcelGel are old.	Ensure that the expiration dates on the buffer strips and ExcelGel have not elapsed.
	Bubbles under the buffer strip.	Ensure that the buffer strips are placed firmly on the gel with no air bubbles trapped beneath them.
	Bubbles under the IPG strip.	Ensure that the IPG strip is placed firmly on the gel with no air bubbles trapped underneath. Stroke the plastic backing of the IPG strip gently with a pair of forceps to remove trapped bubbles.
Buffer strip slides out from under electrode	Incorrect electrode placement.	Ensure that the electrodes are aligned over the centre of the buffer strips before lowering the electrode holder.

Part IV

Visualization and analysis of results

5.0 Visualization of results

Most detection methods used for SDS gels can be applied to second-dimension gels.

Autoradiography and fluorography are the most sensitive detection methods. To employ these techniques, the sample must consist of protein radiolabeled in vivo using either ^{35}S, ^{14}C, ^{3}H, or, in the case of phosphoproteins, ^{32}P. For autoradiographic detection, the gel is simply dried and exposed to X-ray film or a storage phosphor screen. Fluorography is a technique that provides extra sensitivity by impregnating the gel in a scintillant such as PPO (2,4-diphenyloxazole) prior to drying.

Silver staining is the most sensitive nonradioactive method. Silver staining is a complex, multistep process, and many variables can influence the results. High-purity reagents and precise timing are necessary for reproducible, high-quality results. Impurities in the gel and/or the water used for preparing the staining reagents can give poor staining results.

Coomassie staining, although 50-fold less sensitive than silver staining, is a relatively simple method and more quantitative than silver. Coomassie blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

The Hoefer Automated Gel Stainer automates multistep staining processes for increased convenience and reproducibility. Automated protocols #2 and #3, for example, were developed to use the Amersham Pharmacia Biotech PlusOne® Silver Staining Kit, Protein, to silver-stain proteins in SDS gels. This convenient adaptation gives reproducible results and sensitivity below 1 ng per band for most proteins. Protocols #5 and #7 are recommended for Coomassie staining of SDS gels. For complete details, please refer to the Hoefer Automated Gel Stainer Protocol Guide.

5.1 Blotting

Second-dimension gels can be blotted onto a nitrocellulose or PVDF membrane for immunochemical detection of specific proteins or chemical microsequencing.

Note: The plastic backing on ExcelGel products must be removed with a film remover prior to electrotransfer (see 'Ordering information').

5.2 Evaluation

In theory the analysis of up to 15,000 proteins should be possible in one gel; in practice, however, 5,000 detected protein spots means a very good separation. Evaluating high-resolution 2-D gels by a simple comparison of two gels is not always possible. In large studies with patterns containing several thousand spots, it may be almost impossible to detect the appearance of a few new spots or the disappearance of single spots. Image collection hardware and image evaluation software are necessary to detect these differences as well as to obtain maximum information from the gel patterns.

Amersham Pharmacia Biotech ImageMaster 2D Elite Software and 2D Database Software together with the Sharp JX-330 Scanner comprise a system that allows the user to capture, store, evaluate, and present information contained in 2-D gels:

- The Sharp JX-330 Desktop Scanner captures optical information over a range from 0 to 3.0 OD from pixels as small as 42 µm (600 dpi).
- ImageMaster 2D Elite Software provides the essential tools for analyzing complex protein samples separated by 2-D electrophoresis. Protein spots are automatically detected, background is corrected, spot density is quantified, and spots are matched between up to 100 gels. The software can also detect and graphically display quantitative amount changes in spot patterns.
- ImageMaster 2D Database Software adds a database search facility that searches and queries across experiments and images, and analyses experiments for quantitative pattern relationships.

5.3 Standardization of results

The 2-D electrophoresis technique is often used comparatively and thus requires a reproducible method for determining relative spot positions. Because the precast Immobiline DryStrip IPG strips are highly reproducible, the pI of a particular protein can be estimated from its focusing position along a linear pH gradient IPG strip. The second dimension can be calibrated using molecular weight marker proteins loaded to the side of the second-dimension gel. Often there are abundant proteins in the sample for which the pI and molecular weight are known. These proteins can serve as internal standards.

Note: The pI of a protein can depend on its chemical environment and thus can differ depending on the experimental conditions used. Although marker proteins for pI estimation are available, pI estimates based on their use are therefore not necessarily valid.

Troubleshooting

6.0 Troubleshooting 2-D results

Table 26 lists problems that may be encountered in 2-D electrophoresis results, describes the possible causes, and suggests ways to prevent each problem in future experiments.

For troubleshooting problems encountered during the various steps of the 2-D process, refer to the following tables:

- Table 11, page 19. Troubleshooting IPG strip rehydration in Reswelling Tray
- Table 14, page 23. Troubleshooting first-dimension IEF: Multiphor II and Immobiline DryStrip Kit
- Table 17, page 27. Troubleshooting first-dimension IEF: IPGphor
- Table 23, page 33. Troubleshooting vertical second-dimension SDS-PAGE
- Table 25, page 35. Troubleshooting second-dimension SDS-PAGE: Multiphor II flatbed system

TABLE 26. TROUBLESHOOTING 2-D RESULTS

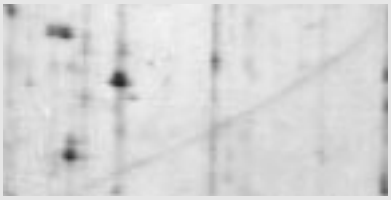
Symptom	Possible cause	Remedy
No distinct spots are visible	Sample is insufficient .	Increase the amount of sample applied.
	Insufficient sample entered the IPG strip due to poor sample solubilization.	Increase the concentration of the solubilizing components in the sample solution. (See section 2.5, "Composition of sample solution.")
	Sample contains impurities that prevent focusing.	Increase the focusing time or modify the sample preparation method. (See "Part I. Sample Preparation.")
	The pH gradient is wrongly oriented.	The pointed end of the Immobiline DryStrip is the acidic end and should point toward the anode (+).
	(Flatbed gel format) IPG strip is placed wrong side down on second-dimension gel.	Ensure that the IPG strip is placed gel-side down (plastic backing upward) on the SDS second-dimension gel.
	Detection method was not sensitive enough.	Use another detection method (e.g., silver staining instead of Coomassie blue staining).
	Failure of detection reagents.	Check expiration dates on staining solutions. Prepare fresh staining solutions.
Individual proteins appear as multiple spots or are missing, unclear, or in the wrong position	Protein carbamylation.	Do not heat any solutions containing urea above 30 ºC, as isocyanate, a urea degradation product, will carbamylate proteins, changing their pI.
	Protein oxidation.	DTT in the rehydration and equilibration solutions keeps the disulphide bonds reduced. For additional protection include an iodoacetamide treatment during equilibration prior to the second-dimension separation. Iodoacetamide alkylates the thiol groups to prevent the reduced proteins from reoxidizing.
Spots are vertically doubled, or "twinned" 	(Vertical gel format) IPG strip is not placed properly.	Ensure that the plastic backing of the IPG strip is against the glass plate on the second-dimension gel.

TABLE 26. TROUBLESHOOTING 2-D RESULTS (continued)

Symptom	Possible cause	Remedy
Distortion of 2-D pattern		
	(Vertical gel format) The top surface of the second-dimension gel is not flat.	Immediately after pouring the gel, overlay the surface with water-saturated butanol.
	(Vertical gel format) Uneven polymerization of gel due to incomplete polymerization, too rapid polymerization, or leakage during gel casting.	Degas the gel solution. Polymerization can be accelerated by increasing by 50% the amount of ammonium persulphate and TEMED used. Polymerization can be slowed by decreasing by 33% the amount of ammonium persulphate and TEMED used. Ensure that there is no leakage during gel casting.
	(Flatbed gel format) Moisture on the surface of the second-dimension gel.	Allow ExcelGel to dry for about 5 minutes after removing plastic cover and before applying buffer strips and IPG strip.
	(Flatbed gel format) IPG strip not removed during electrophoresis.	Remove the IPG strip and application pieces from the second-dimension gel when the bromophenol blue dye from has moved away from the IPG strip by 4–6 mm.
	(Flatbed gel format) Air bubbles under the second-dimension gel cause uneven migration due to poor heat transfer.	Ensure that no bubbles are trapped under the second-dimension gel during placement on the cooling plate.
	(Flatbed gel format) Water drops or pieces of buffer strip on the surface of the second dimension gel.	Take care that nothing is dropped or splashed onto the surface of the second-dimension gel.
Horizontal streaking or incompletely focused spots		
	Sample not completely solubilized prior to application.	Be sure that the sample is completely and stably solubilized. *Note:* Repeated precipitation-resolubilization cycles produce or increase horizontal streaking. See section 2.5, "Composition of the sample solution," for general guidelines for sample solubilization.
	Sample is poorly soluble in rehydration solution.	Increase the concentration of the solubilizing components in the rehydration solution. (See section 3.4, "IPG strip rehydration solution.") Increase concentration of IPG Buffer.

TABLE 26. TROUBLESHOOTING 2-D RESULTS (continued)

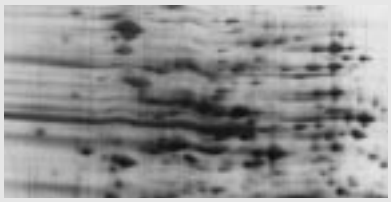
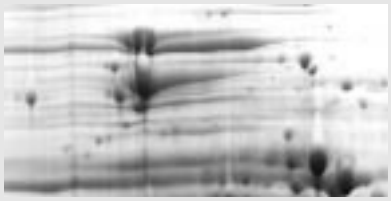
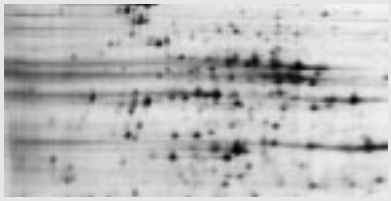
Symptom	Possible cause	Remedy
Horizontal streaking or incompletely focused spots (continued)		
	Interfering substances. Non-protein impurities in the sample can interfere with IEF, causing horizontal streaking in the final 2-D result, particularly toward the acidic side of the gel.	Modify sample preparation to limit these contaminants. (See section 2.4, "Removal of contaminants that affect 2-D results.")
	Ionic impurities in sample.	Reduce salt concentration to below 10 mm by dilution or desalt the sample by dialysis. Precipitation with TCA and acetone and subsequent resuspension is another effective desalting technique that removes lipids, nucleotides and other small molecules. *Note:* Specific and non-specific losses of proteins can occur with dialysis, gel chromatography, and precipitation/resuspension of samples. If the sample preparation cannot be modified, the effect of ionic impurities can be reduced by modifying the IEF protocol. Limit the voltage to 100–150 V for 2 hours, then resume a normal voltage step program. This pre-step allows the ions in the sample to move to the ends of the IPG strip.
	Ionic detergent in sample.	If the ionic detergent SDS is used in sample preparation, the final concentration must not exceed 0.25% after dilution into the rehydration solution. Additionally, the concentration of the non-ionic detergent present must be at least 8 times higher than the concentration of any ionic detergent to ensure complete removal of SDS from the proteins.
	High sample load.	Load less sample. Micropreparative separations require clean sample. Modify sample preparation to limit contaminants. (See section 2.4, "Removal of contaminants that affect 2-D results.") Program a low initial voltage and increase voltage gradually. Extend focusing time.
	Underfocusing. Focusing time was not long enough to achieve steady state focusing.	Prolong focusing time.
	Overfocusing. Extended focusing times (over 100,000 Vh) may result in electroendosmotic water and protein movement, which can produce horizontal smearing.	Reduce focusing time.

TABLE 26. TROUBLESHOOTING 2-D RESULTS (continued)

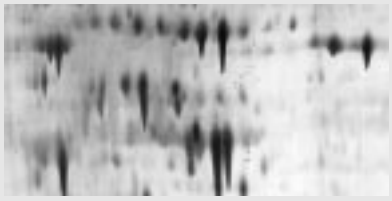
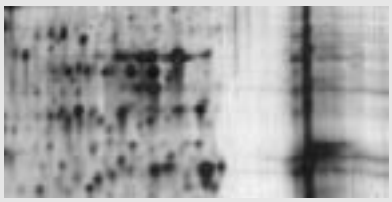
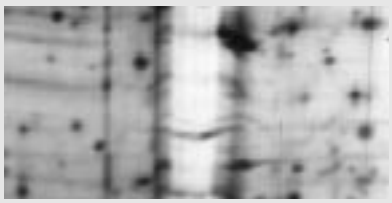
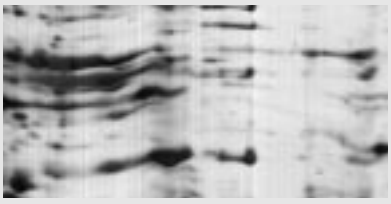
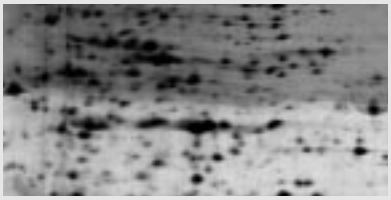
Symptom	Possible cause	Remedy
Horizontal stripes across gel	Impurities in agarose overlay or equilibration solution.	Prepare fresh agarose overlay and equilibration solution.
Prominent vertical streak at the point of sample application (when loading IPG strips using sample cups)	(Flatbed gel format) Sample aggregation or precipitation.	Dilute the sample and apply as a larger volume. Program a low initial voltage and increase voltage gradually.
Vertical streaking	Insufficient equilibration.	Prolong equilibration time.
	(Flatbed gel format) Electroendosmosis.	Add 30% glycerol and 6 M urea to the SDS equilibration buffer. Place application pieces at the end of the strips during second-dimension electrophoresis to absorb excess water.
	Second-dimension buffer solutions prepared incorrectly.	Prepare fresh solutions.
	Insufficient SDS in SDS electrophoresis buffer.	Use 0.1% w/v SDS.
Vertical gap in 2-D pattern 	Impurities in sample.	Modify sample preparation. (See section 2.4, "Removal of contaminants that affect 2-D results.")
	Impurities in rehydration solution components.	Use only high-quality reagents. De-ionize urea solutions

TABLE 26. TROUBLESHOOTING 2-D RESULTS (continued)

Symptom	Possible cause	Remedy
	Bubble between IPG strip and top surface of second-dimension gel.	Ensure that no bubbles are trapped between the IPG strip and the top surface of the second-dimension gel.
	(Flatbed gel format) Urea crystals on the surface of the IPG strip.	Allow residual equilibration solution to drain from the IPG strip before placing the strip on the second-dimension gel.
	(Flatbed gel format) Bubbles under the IPG strip.	Ensure that the IPG strip is placed firmly on the gel with no air bubbles trapped underneath. Stroke the plastic backing of the IPG strip gently with a pair of forceps to remove trapped bubbles.
Vertical regions of poor focusing		
	The IPG strip was not fully rehydrated.	Ensure that the IPG strips are rehydrated with a sufficient volume of rehydration solution. Remove any large bubbles trapped under the IPG strip after rehydration solution is applied. Check that the rehydration solution is evenly spread along the entire length of the IPG strip.
Poor representation of higher molecular weight proteins	Proteolysis of sample.	Prepare sample in a manner that limits proteolysis and/or use protease inhibitors. (See section 2.2, "Protection against proteolysis.")
	Insufficient equilibration.	Prolong equilibration time.
	Poor transfer of protein from IPG strip to second-dimension gel.	Employ a low current sample entry phase in the second-dimension electrophoresis run.
	Poor entry of sample protein during rehydration.	Use recommended volume of rehydration solution. (See Tables 10 and 15.)
Point streaking		
	(Silver staining). Dirty plates used to cast gel or particulate material on the surface of the gel. DTT and other thiol reducing agents exacerbate this effect.	Properly wash glass plates. Scavenge any excess or residual thiol reducing agent with iodoacetamide before loading the IPG strips onto the second-dimension gel.
Background smear toward bottom of gel	(Silver or Coomassie blue staining) Staining of carrier ampholytes.	Use IPG Buffer as carrier ampholyte mixture. Reduce concentration if necessary.
Background smear toward top of gel	(Silver staining) Nucleic acids in sample.	Add DNase and RNase to hydrolyze nucleic acids. *Note:* The proteins DNase and RNase may appear on the 2-D map.
High background in top region of gel		
	Protein contaminant in SDS electrophoresis buffer or dirty electrophoresis unit.	Make fresh SDS electrophoresis buffer. Clean electrophoresis unit.

Appendix: Solutions

A. Lysis solution

(8 M urea, 4% CHAPS, 40 mM Tris (base), 40 ml)

	Final concentration	Amount
Urea (FW 60.06)	8 M[1]	19.2 g
CHAPS[2]	4% (w/v)	1.6 g
Tris base (FW 121.1)	40 mM	0.194 g
Double distilled H_2O		to 40 ml

Prepare fresh or store in aliquots at –20 °C.

[1] If necessary, the concentration of urea can be increased to 9 or 9.8 M.

[2] Other detergents (Triton X-100, NP-40, and other non-ionic or zwitterionic detergents) can be used instead of CHAPS.

Note: Protease inhibitors and/or reductants may be added if necessary.

B. Rehydration stock solution without IPG Buffer[1]

(8 M urea, 2% CHAPS, bromophenol blue, 25 ml)

	Final concentration	Amount
Urea (FW 60.06)	8 M[2]	12 g
CHAPS[3]	2% (w/v)	0.5 g
Bromophenol blue	trace	(a few grains)
Double distilled H_2O		to 25 ml

Store in 2.5 ml aliquots at –20 °C.

[1] DTT and IPG Buffer are added just prior to use: Add 7 mg DTT per 2.5 ml aliquot of rehydration stock solution. See Table 9 for the appropriate volume of IPG Buffer to use. If loading sample by inclusion in the rehydration solution, sample is also added to the 2.5 ml aliquot of rehydration solution just prior to use.

[2] If necessary, the concentration of urea can be increased to 9 or 9.8 M.

[3] Other detergents (Triton X-100, NP-40, and other non-ionic or zwitterionic detergents) can be used instead of CHAPS.

C. Rehydration stock solution with IPG Buffer[1]

(8 M urea, 2% CHAPS, 0.5% or 2% IPG Buffer,[2] bromophenol blue, 25 ml)

	Final concentration	Amount
Urea (FW 60.06)	8 M[3]	12 g
CHAPS[4]	2% (w/v)	0.5 g
IPG Buffer (same pH range as the IPG strip)	0.5% or 2% (v/v)[5]	125 or 500 µl[6]
Bromophenol blue	trace	(a few grains)
Double distilled H_2O		to 25 ml

Store in 2.5 ml aliquots at –20 °C.

[1] DTT is added just prior to use: 7 mg DTT per 2.5 ml aliquot of rehydration stock solution. If loading sample by inclusion in the rehydration solution, sample is also added to the 2.5 ml aliquot of rehydration solution just prior to use.

[2] Either of two IPG Buffer concentrations is recommended depending on the IEF system used and the pH range of the IPG strip. Refer to Table 9.

[3] If necessary, the concentration of urea can be increased to 9 or 9.8 M.

[4] Other detergents (Triton X-100, NP-40, and other non-ionic or zwitterionic detergents) can be used instead of CHAPS.

[5] Selection of IPG Buffer concentration is based on IEF system used and pH range of the IPG strip. Refer to Table 9.

[6] Use 125 µl IPG Buffer for a 0.5% concentration and 500 µl IPG Buffer for a 2% concentration.

D. SDS equilibration buffer[1]

(50 mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 200 ml)

	Final concentration	Amount
1.5 M Tris-Cl, pH 8.8 (see solution F)	50 mM	6.7 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophenol blue	trace	(a few grains)
Double distilled H_2O		to 200 ml

Store in 40 ml aliquots at –20 °C.

[1] This is a stock solution. Prior to use DTT or Iodoacetamide is added. See section 4.2.2.

E. Monomer stock solution

(30% acrylamide, 0.8% N,N'-methylenebisacrylamide, 200 ml)

	Final concentration	Amount
Acrylamide (FW 71.08)	30%	60.0 g
N,N'-methylenebisacrylamide (FW 154.17)	0.8%	1.6 g
Double distilled H_2O		to 200 ml

Filter solution through a 0.45 µm filter.
Store at 4 °C away from light.

F. 4X Resolving gel buffer

(1.5 M Tris-Cl pH 8.8, 1000 ml)

	Final concentration	Amount
Tris base (FW 121.1)	1.5 M	181.5 g
Double distilled H_2O		750 ml
HCl (FW 36.46)		adjust to pH 8.8
Double distilled H_2O		to 1000 ml

Filter solution through a 0.45 µm filter.
Store at 4 °C.

G. 10% SDS

	Final concentration	Amount
SDS (FW 288.38)	10% (w/v)	5.0 g
Double distilled H_2O		to 50 ml

Filter solution through a 0.45 µm filter.
Store at room temperature.

H. 10% Ammonium persulphate

	Final concentration	Amount
Ammonium persulphate (FW 228.20)	10%	0.1 g
Double distilled H_2O		to 1.0 ml

Fresh ammonium persulphate "crackles" when water is added. If it does not, replace it with fresh stock. Prepare just prior to use.

I. Gel storage solution

(0.375 M Tris-Cl pH 8.8, 0.1% SDS 200 ml)

	Final concentration	Amount
4X resolving gel buffer (see solution F)	1X	50 ml
10% SDS (see solution G)	0.1%	2 ml
Double distilled H_2O		to 200 ml

Store at 4 °C.

J. SDS electrophoresis buffer[1]

(25 mM Tris, 192 mM glycine, 0.1% SDS, 5 liters)

	Final concentration	Amount
Tris base (FW 121.1)	25 mM	15.1 g
Glycine (FW 75.07)	192 mM	72.1 g
SDS (FW 288.38)	0.1% (w/v)	5.0 g
Double distilled H_2O		to 5000 ml

Store at room temperature.

[1] Because the pH of this solution need not be checked, it can be made up directly in large reagent bottles marked at 5.0 liters. 20 liters can be made up at a time and stored at room temperature.

K. Agarose sealing solution

	Final concentration	Amount
SDS electrophoresis buffer (see solution J)		100 ml
Agarose (NA or M)	0.5%	0.5 g
Bromophenol blue	trace	a few grains

Add all ingredients into a 500 ml Erlenmeyer flask. Swirl to disperse. Heat in a microwave oven on low until the agarose is completely dissolved. Do not allow the solution to boil over. Dispense 2 ml aliquots into screw-cap tubes and store at room temperature.

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Ordering information

First dimension

Multiphor II/Immobiline DryStrip Kit focusing system and accessories

18-1018-06	Multiphor II Electrophoresis Unit
18-1004-30	Immobiline DryStrip Kit, complete
80-6371-84	Immobiline DryStrip Reswelling Tray
18-1130-05	EPS 3501 XL Power Supply
18-1102-77	MultiTemp III Thermostatic Circulator, 115 V
18-1102-78	MultiTemp III Thermostatic Circulator, 230 V
18-1004-35	Sample cups (60/pk)
18-1004-40	IEF electrode strips (100/pk)

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80-6414-02	IPGphor isoelectric focusing unit (order strip holders separately)
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	7 cm	*11 cm*	*13 cm*	*18 cm*
6/pk	80-6416-11	80-6416-30	80-6416-49	80-6416-68
1/pk	80-6416-87	80-6417-06	80-6417-25	80-6417-44

Immobiline DryStrip gels (12/pk)

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pH 4–7 L	17-6001-10	18-1016-60	17-6001-13	17-1233-01
pH 6–11 L	17-6001-34	17-6001-35	17-6001-36	17-6001-37
pH 3–10 L	17-6001-11	18-1016-61	17-6001-14	17-1234-01
pH 3–10 NL	17-6001-12	N/A	17-6001-15	17-1235-01

IPG Buffer, 1 ml

17-6000-86	pH 4–7 L
17-6000-87	pH 3–10 L
17-6000-88	pH 3–10 NL
17-6001-78	pH 6–11 L

Pharmalyte, 25 ml

17-0453-01	pH 5–8
17-0455-01	pH 8–10.5

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80-6150-30 Spacer, 1.5 mm (2/pk)
80-6149-35 SE 260 Mighty Small II Vertical Unit, complete, for 2 slab gels
80-6146-12 SE 235 Mighty Small 4-Gel Caster, complete
80-6146-50 SE 245 Mighty Small Dual Gel Caster
80-6223-83 Thin fluorescent rulers (2/pk)
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Hoefer SE 600 vertical unit and accessories

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80-6180-13 Spacer, 1.5 mm, 1 cm wide (2/pk)
80-6180-89 Spacer, 1.5 mm, 2 cm wide (2/pk)
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80-6182-79 SE 615 Multiple Gel Caster for 2 to 10 gels (glass plate size: 18 × 16 cm)

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80-6330-61 DALT Multiple Gel Caster for 23 gels
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80-6067-46 DALT Cassette with 1.5 mm spacers
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80-6197-99 SG 50 Gradient Maker, 50 ml total volume
80-6196-09 SG 100 Gradient Maker, 100 ml total volume
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80-1129-46 IEF sample application pieces (200/pk)
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PlusOne electrophoresis chemicals and reagents

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17-1314-01	CHAPS, 1 g
17-1315-01	Triton X-100, 500 ml
17-1318-01	Dithiothreitol (DTT), 1 g
17-1329-01	Bromophenol blue, 10 g
80-1130-01	Ultrodex granulated gel, 50 g
17-1335-01	IPG Cover Fluid, 1,000 ml
17-1302-01	Acrylamide PAGE (acrylic acid <0.05%), 250 g
17-1302-02	as above, 1 kg
17-1300-01	Acrylamide IEF (acrylic acid <0.002%), 250 g
17-1300-02	as above, 1 kg
17-1303-01	Acrylamide PAGE 40% solution, 1,000 ml
17-1301-01	Acrylamide IEF 40% solution, 1,000 ml
17-1304-01	N,N'-methylenebisacrylamide, 25 g
17-1306-01	N,N'-methylenebisacrylamide 2% solution, 1,000 ml
17-1321-01	Tris, 500 g
17-1313-01	SDS, 100 g
17-1325-01	Glycerol (87%), 1 L
17-1311-01	Ammonium persulphate, 25 g
17-1312-01	TEMED, 25 ml
17-1323-01	Glycine, 500 g
17-0422-01	Agarose M, 10 g
17-0554-01	Agarose NA, 10 g

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27-0323-01	Ribonuclease I "A" (RNase A), 100 mg

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80-6343-34 Protocol Guide, Hoefer Automated Gel Stainer

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